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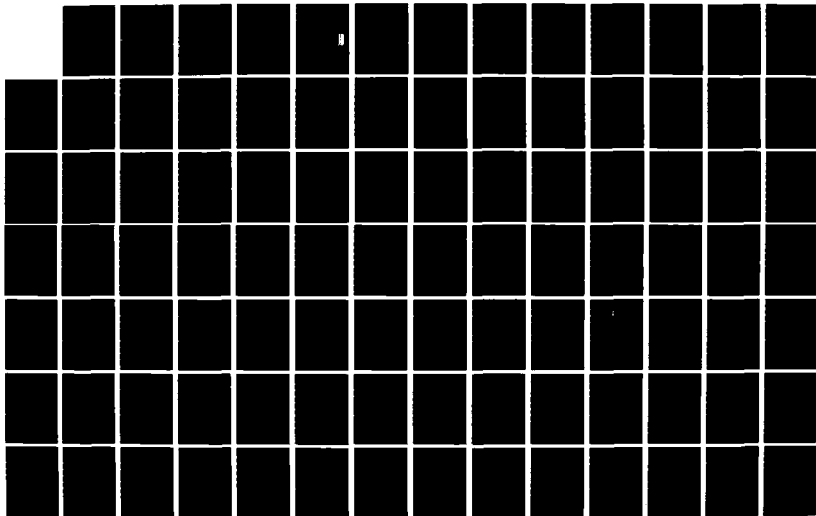
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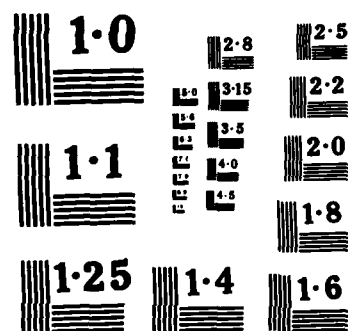
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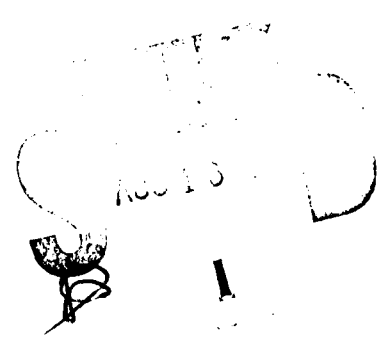
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CELL COMMUNICATION DURING AGGREGATION AND DEVELOPMENT  
OF THE CELLULAR SLIME MOULD DICTYOSTELIUM DISCOIDEUM

A thesis submitted to the Board of the  
Faculty of Biological and Agricultural  
Sciences, The University of Oxford, in  
partial fulfilment of the requirements  
for the degree of Doctor of Philosophy.

by

Henry S. Tillinghast, Jr.



Merton College, Oxford

June, 1985

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## ABSTRACT

The role for folates and pterins as potential morphogens during tipped aggregate formation was studied. A revised folate binding assay was employed to demonstrate the presence of folate receptors on vegetative Dictyostelium discoideum amoebae. These receptors are present at 45,000 per cell and function as a single class that bind folate and deaminofolate (DAFA) with equal affinity (dissociation constant = 300nM). A pool of receptors was found attached to the cytoskeletons of vegetative amoebae and these receptors (10,000 sites/cell, Kd 480nM) remain associated with the cytoskeleton through the first 12hr of development. A role for the cytoskeleton in receptor modulation during signal transduction is postulated.)

Competitive binding studies with 21 folate and pterin analogues revealed that the folate receptor shows a high degree of specificity for the intact folate moiety. Minor substitutions on the pteridine ring do not significantly disrupt binding, however, the folate receptor is sensitive to modifications on the N<sup>10</sup> position and the benzene ring of the folate molecule, suggesting important receptor/ligand interactions at these locations.

The folate-induced, rapid and transient accumulation of actin in the Triton X-100 insoluble cytoskeletons correlates with tipped aggregate formation in developing Dictyostelium cells. A surprising role for L-monapterin as an active chemoattractant during tipped aggregate formation is described. L-monapterin also induces an actin response at this developmental stage and antagonist studies suggest that the L-monapterin response is mediated by a separate pterin receptor. The evolutionary significance of multiple acrasin and acrasinase systems in cellular slime mould development is discussed.

Calcium mobilization appears essential in signal transduction of the folate-induced actin response. However, the calcium-immobilizing drug, TMB-8, used in this study is a potent inhibitor of cellular respiration. The 12hr actin response does not involve the "second messenger" cyclic GMP. A potential role for the bifurcated diacylglycerol/inositol triphosphate signal transduction mechanism in the cyclic GMP- and calcium-mediated responses is postulated.

A developmental mutant, incapable of forming tipped aggregates, was employed to study the role of cell-cell contact in signal transduction and tipped aggregate formation. This mutant may produce only prespore cells. The mutant possesses the genetic capability to produce prespore and spore specific proteins yet can only be induced to complete development when in direct contact with wild-type cells.

The role of cell interaction and developmental morphogens in cellular slime mould development is discussed.

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## *Figures*

STF	slug turning factor
stm	carrying mutation at a "streamer" locus
TEMED	N,N,N',N'-tetramethylethylenediamine
TMB-8	(8-diethylamino)octyl-3,4,5-trimethoxy benzoate, HCl
Tris	Tris-(hydroxymethyl)-methyllamine
U	one unit of DIF induces 1% stalk cells in a standard 2ml assay
UPS	upper pad solution

KMMP	potassium, manganese, magnesium, phosphate
KP6	potassium phosphate buffer, 20mM, pH 6.0
LPS	lower pad solution
$\frac{M}{r}$	molecular ratio
MTX	methotrexate, amethopterin
N <sup>10</sup> -CH <sub>3</sub> -folate	N <sup>10</sup> -methyl-folate
NC4	wild-type <u>D. discoideum</u> strain
NEPHGE	non-equilibrium pH gradient electrophoresis
NP-40	Nonidet P-40 detergent
NP368	"streamer" mutant of <u>D. discoideum</u>
NP377	"streamer" mutant of <u>D. discoideum</u>
NP550	"brainy" mutant of <u>D. discoideum</u>
NP551	"brainy" mutant of <u>D. discoideum</u>
NTG	N-methyl-N'-nitro-N-nitrosoguanidine
p-ABA	para-aminobenzoic acid
p-ABA-glu	para-aminobenzoyl glutamate
PPO	2,5-diphenyloxazole
PV	prespore vesicles
R <sub>f</sub>	relative mobility
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	seconds
S.E.M.	standard error of the mean
SM	standard medium (nutrient agar)
SORPHOS	Sorenson's phosphate buffer, 17mM, pH 6.15
SS	standard salts solution
SPIF	spore protein inducing factor

## ABBREVIATIONS

A 23187	calcium ionophore
AUFS	absorption units full scale
AX3	axenic mutant of <u>D. discoideum</u>
cAMP	adenosine 3':5'-cyclic monophosphate
[ <sup>3</sup> H]cAMP	[8- <sup>3</sup> H]adenosine 3':5'-cyclic monophosphate
cGMP	guanosine 3':5'-cyclic monophosphate
COMP	competitor
ConA	concanavalin A
cytoskeleton	residue from cells after Triton X-100 extracton
DAFA	2-deamino-2-hydroxy-folic acid
DES	diethylstilboesterol
DIF	differentiation inducing factor
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid
F-actin	filamentous configuration of actin protein
G-actin	globular configuration of actin protein
xg	relative centrifugal force
glorin	N-propionyl- $\gamma$ -L-glutamyl-L-ornithine- $\delta$ -lactam ethyl ester
[ <sup>3</sup> H]FA	[7,9,3',5'- <sup>3</sup> H]folic acid
[ <sup>3</sup> H]DAFA	[7,9,3',5'- <sup>3</sup> H]-2-deamino-2-hydroxy folic acid
HPLC	high performance liquid chromatography
hr	hour
I <sub>50</sub>	competitor concentration that inhibits ligand binding by 50%
k	kilo
K <sub>d</sub>	dissociation constant

## *Abbreviations*

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# *Chapter 1*

## 1 INTRODUCTION

### 1.1 THE CELLULAR SLIME MOULDS

The first cellular slime mould species, Dictyostelium mucoroides was discovered by the German mycologist Brefeld in 1869. The taxonomy, life cycles, and physical characteristics of the early known species were first described by E.W. Olive (1902) (see Bonner, 1982a). Yet today, the more recent taxonomic studies (Raper, 1973, 1982; L.S. Olive, 1975) still do not agree on the classification of this organism. Raper prefers to consider them fungi whereas Olive unites cellular slime moulds with protozoa. Any taxonomic classification system will create artificial boundaries, yet there is probably a continuum between protozoa and fungi and slime moulds may have evolved within the continuum. Robertson (1977) is somewhat more pessimistic and has stated that "it is a bit difficult to avoid the feeling that the slime moulds represent a cul-de-sac in evolution". Irrespective of the taxonomic dilemma, this unique eukaryote with its unicellular growth phase and the multicellular developmental phase is an amenable model system, applicable to a broad spectrum of biochemical studies from chemotaxis and signal transduction to developmental pattern formation.

#### 1.1.1 The Life Cycle of Dictyostelium discoideum

Dictyostelium discoideum, discovered by Raper (1935), has become the most studied species of the cellular slime moulds. Workers have been attracted to this species for several reasons. D. discoideum produces a large fruiting body (2-3mm high) and consequently these fruiting bodies and the other developmental forms are large enough to be easily manipulated during experimental studies. The short developmental cycle (24hr) and the ease of maintenance and growth of this simple eukaryote provide many advantages over the more fastidious and fragile

eukaryotic cells. As a model for studying chemotaxis and signal transduction the vegetative slime mould amoebae are extremely similar to mammalian leucocytes and other motile eukaryotic cells. Another feature common to all cellular slime moulds, in contrast to other plants and animals, is that the cellular growth phase is distinct from the developmental phase of the life cycle. This feature allows workers to study the process of development and differentiation without the complicating features of cell and tissue growth. Additionally, the clear division of labour, evident in the two presumptive cell types that are present at two distinct locations during the migration stage (the slug stage) (section 1.1.1 D), has attracted many workers interested in cytodifferentiation and morphogenetic development. The stages of the Dictyostelium discoideum life cycle are discussed in more detail in the sections to follow (figure 1.1).

#### A Vegetative Stage

During the vegetative phase of growth the amoebae inhabit soil, humus, and animal dung often within forest litter and normally feed on the soil bacteria by phagocytosis. Unicellular growth and division by binary fission continues as long as a bacterial food source is available. In the laboratory the food source most frequently supplied is either Klebsiella aerogenes or Escherichia coli. If the food source becomes scarce or the humidity of the soil environment declines, then the solitary amoebae will enter a social phase of multicellular development.

#### B Aggregation Stage

Upon starvation, a few individual amoebae within the apparently homogeneous population begin to emit pulses of the chemical attractant cAMP which elicits two responses from the surrounding cells. First, the cells move chemotactically toward the signal source and secondly, the

Figure 1.1      Life Cycle of Dictyostelium discoideum

# LIFE CYCLE OF DICTYOSTELIUM

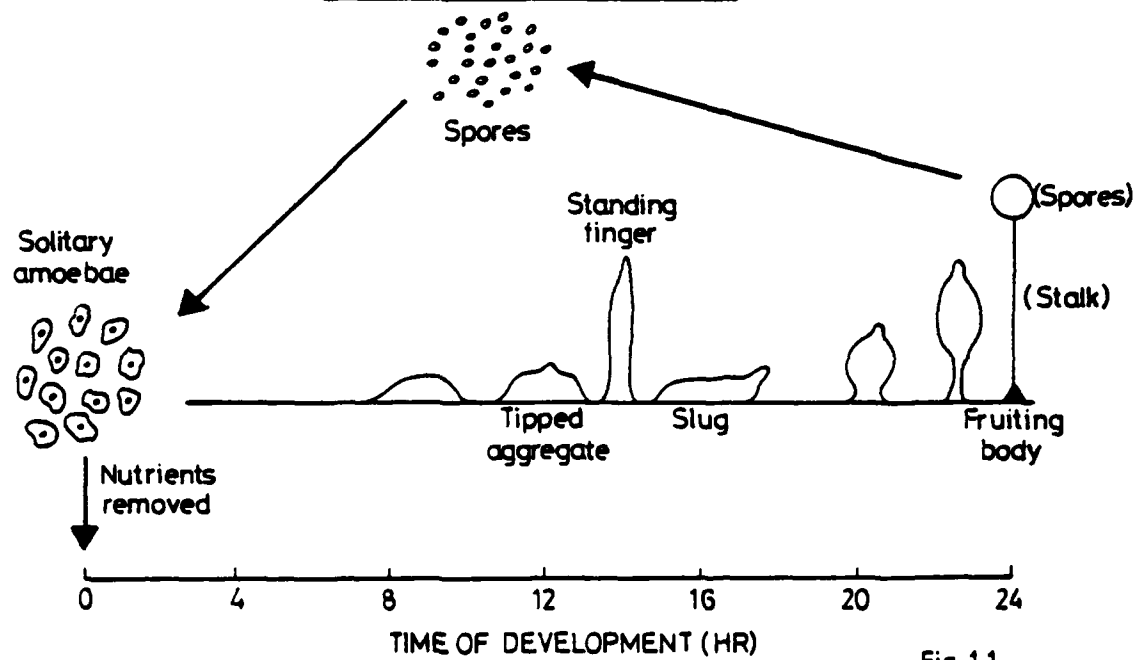


Fig. 1.1

attracted cells relay the message by emitting a pulse of cAMP themselves. This message-relay process produces successive waves of chemoattractant that pass outward from the signalling centre. In response, waves of attracted cells move inward and eventually the waves dissipate into streams of migrating amoebae which enter a multicellular aggregate at the signalling centre.

#### C Tipped Aggregate Formation

The central aggregate is first evident as a hemispherical mound which rapidly forms a tip (approximately 12hr after the onset of starvation). In the tipped aggregate, there is the first evidence of division of labour. Much evidence suggests that at the time of tip formation, the initial fate of individual cells is established (Sussman and Osborne, 1964; Takeuchi et al., 1977; West and McMahon, 1979; Mehdy et al., 1983; Tasaka et al., 1983; Morrissey et al., 1984). At this stage of development the population of mRNAs doubles in complexity (from 4000 to 8000 species) and it is estimated that several thousand new genes are activated (Landfear and Lodish, 1980). The tip on this developmental aggregate functions as an organizer region and controls morphogenetic movement at all remaining stages of D. discoideum development (Rubin and Robertson, 1975; Robertson, 1977).

#### D Migration Stage

Within two hours of formation, the tipped aggregate rises into the air as an elongated cylinder called the "standing finger". If environmental conditions are favourable, this cylinder of cells will assume a horizontal orientation along the substratum and migrate. The migrating "slug" or pseudoplasmodium then crawls through the cavities in the soil and is attracted by light and heat to the soil surface. Bonner (1969) observed that spore dispersal must be of key importance in

the natural selection of soil organisms, and the formation of a slug and its migration to "a better place in the sun" is therefore an important adaptation for more effective spore dispersal.

The slug stage has received much research interest, since in this stage the two presumptive cell types (prestalk and prespore cells) are positionally segregated. The anterior one-fifth of the slug contains the prestalk cells that eventually form the tapered stalk of the mature fruiting body. The posterior four-fifths of the slug constitutes the prespore zone and these cells form the encapsulated spores contained within the sori at the apex of the fruiting body (Raper, 1940).

The presence of the two morphogenetic fields (Kay and Jermyn, 1983) within the migrating slug have prompted intense interest in this particular developmental stage and many theoretical models have been proposed to explain the slug cell pattern (Meinhardt, 1983; for review MacWilliams and Bonner, 1979; Morrissey, 1982). Once the migrating slug reaches the soil surface, overhead light and low humidity induce the slug to form a "Mexican hat-shaped" aggregate which is symmetrical about the vertical axis and to enter the final phase of fruiting body formation.

#### E Culmination

At the beginning of culmination leading to fruiting body formation, the prestalk cells at the tip of the aggregate begin to form stalk cells through a process of progressive vacuolation and deposition of cellulose cell walls (Bonner, 1944; Bonner, 1982a). The apical stalk cells, through a reverse fountain movement, push down through the prespore zone, and in doing so, the tipped aggregate lowers and bulges laterally to form the "Mexican hat stage". Once the elongating stalk reaches the base, a circle of cells from the posterior of the slug also differentiate into stalk cells to form the basal disc (hence D.

discoideum). Stalk cells continue to add to the apex of the stalk and as the stalk elongates, the whole prespore mass rises into the air. As the prespore cells migrate to the stalk apex, they gradually differentiate into cellulose-encased, oval-shaped spores. The final fruiting body has a slender, tapering stalk with a terminal, often lemon-shaped, spore mass (Bonner, 1982a). The fruiting body, 2-3mm in height, may be an optimum height for successful dispersal of spores which can adhere to passing invertebrates such as nematodes, earthworms, and insect larvae. The spores could also be carried in the gut of invertebrates as is known to occur for the spores of many fungi (Pherson and Beatie, 1979; Bonner, 1982a). Suthers (1985) has reported that widespread dispersal of slime mould spores may be possible by passage through ground-feeding migratory birds.

The germination of a single spore in a new, suitable environment will produce the parent of a new amoebal population and will initiate another life cycle.

#### 1.1.2 Dictyostelium discoideum: A Model for Studying Morphogenetic Development

Dictyostelium discoideum is the simplest possible model eukaryotic organism one can employ to study cytodifferentiation and morphogenetic development. Biologists and biochemists generally agree that communication events with morphogens as the messengers and cells as the receivers in some way lead to cell differentiation and morphogenetic development. With many cell types and many potential messengers in advanced multicellular eukaryotes, identifying such communication links is at present a very difficult task. In slime moulds we have minimum complexity, so such communication events should be more easily identified. Three chemical signals (morphogens) have been provisionally identified, cAMP,  $\text{NH}_3$ , and DIF (Sussman and Schindler, 1978; Morrissey, 1983; Kay and Jermyn, 1983; Gross et al., 1983; Kopachik et al., 1983,

1985), and other putative morphogens such as folates, STF, SPIF, arachidonic acid, and carboxylic acids have been proposed (Williams, 1982; Sussman, 1982; Fisher et al., 1984; McConaghy et al., 1984; Williams et al., 1984). The communication events involved in the differentiation of the two cell types within this simple organism are becoming rather complex. In the remainder of this introduction I will review the role of cell-cell communication in cellular slime moulds and discuss briefly several published models that attempt to explain morphogenetic development and signal transduction in Dictyostelium.

## 1.2 COMMUNICATION IN THE CELLULAR SLIME MOULDS

Before one can study communication events in any organism, it seems appropriate to first determine the components that may mediate such an event. The concept of communication is certainly of interest to many disciplines and the mathematics based "theories of communication" are an attempt to establish a common ground among the disciplines. Any attempts to establish such a commonality are complicated by the diversity of the messages and the nature of the receivers (Cherry, 1980). While attempting to avoid diversions beyond the scope of this thesis, I have drawn upon the knowledge of communication in other disciplines and have employed a general communication model as a foundation for reviewing communication events in Dictyostelium. Figure 1.2 correlates the general diagram of a theoretical communication event (Shannon and Weaver, 1963) with the more specific case seen in cell-cell communication in Dictyostelium. This rather simplistic communication scheme is presented as a basis for discussing the general components involved in cell-cell communication in Dictyostelium. In the following section each component of this hypothetical communication model is briefly described, then in the sections to follow evidence to support the existence of the major components is reviewed in more depth.

Figure 1.2     The Components of Cell-Cell Communication  
                  in Dictyostelium

The components of cell-cell communication in Dictyostelium (b) are adapted from the more general communication scheme (a) (Shannon and Weaver, 1963).

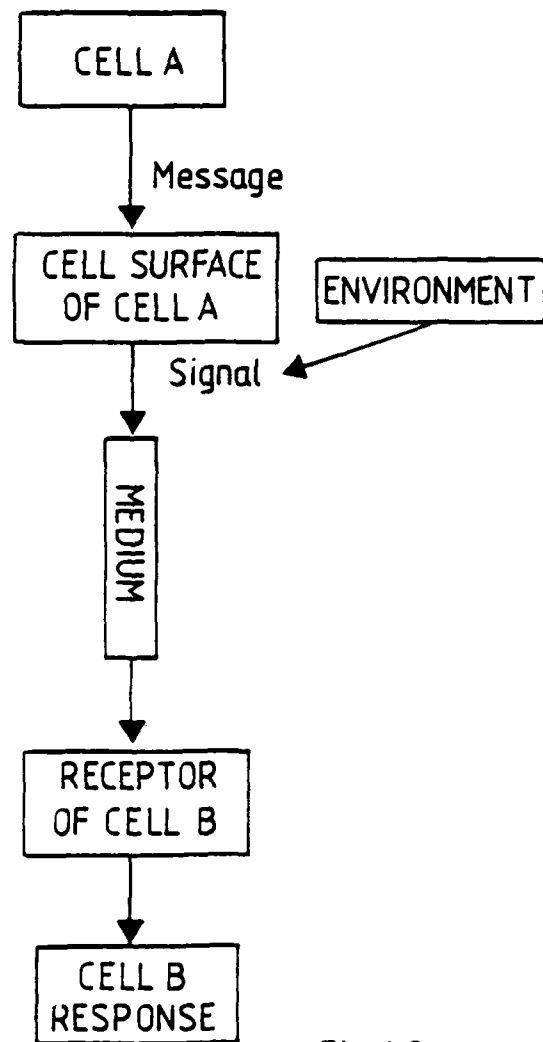
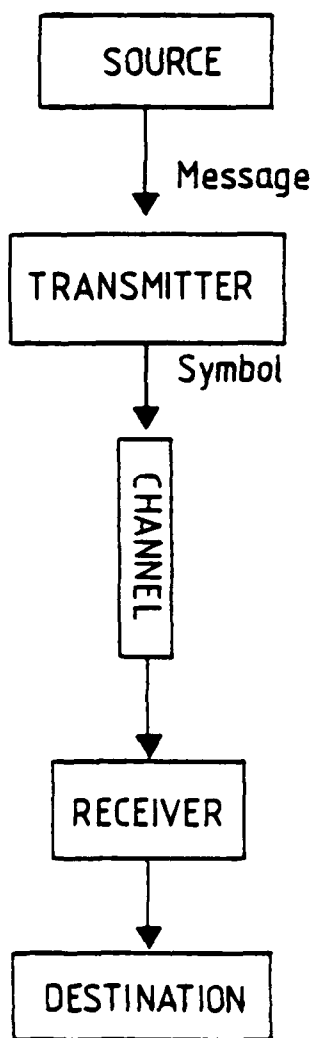


Fig. 1.2

### 1.2.1 The Communication Components Involved in Cellular Slime Mould Development

#### A The Message Source

The initial step in any communication event is the formulation of a message and transmission of that message from the source. In cellular slime moulds, the source of that message is either the environment or specific cells. During aggregation the cell environment (in the form of lack of nutrients) very likely initiates the communication event by inducing the first individual amoebae to emit cAMP pulses. Upon receipt of the message via the signal molecule (cAMP) the receiver cells then relay (re-transmit) the message and communication continues and aggregation occurs. In the developing multicellular aggregate, the initial source of information is most likely either the environment or one of the two cell types (prestalk or prespore cells). Prior to tip formation, when only one cell type is present, the cells retain the choice of entering either the prestalk or prespore pathway of differentiation. Therefore, the signals that initiate cell-type specific differentiation events are apparently environmental or position dependent (Meinhardt, 1983). Once cell-type differentiation has occurred, then specific cell types can initiate other cell-type specific differentiation signals or signals that dictate morphogenetic development (e.g. chemotactic signals, Meinhardt, 1983; Morrissey, 1982).

#### B The Signal

If the source of information is the external environment, possibly indicative of the cell's position within an aggregate, then this information will be channeled directly through the medium without

Geltosky et al. (1976, 1979) have described a developmentally regulated 150kdalton glycoprotein that appears to function in cell adhesion between 6 and 18hr of development. Fluorescent-labelled antibodies raised against the glycoprotein bound more at sites of cell to cell contact in aggregates than on the remainder of the cell surface (Geltosky et al., 1980). A cell-type specific function for gpl50 in prespore cells has been suggested (Lam et al., 1981).

#### C Lectins Involved in Cell Adhesion

Slime mould cells appear to possess developmentally regulated cell surface lectins since whole cells and cell extracts agglutinate formalized sheep erythrocytes (Rosen et al., 1973) and antibodies against purified lectins bind to cell-surface components (Chang et al., 1975, 1977). Three slime mould species possess distinct yet similar lectins: D. discoideum, discoidin; D. purpureum, purpurin; Polysphondylium pallidum, pallidin (for review, Barondes et al., 1982). These carbohydrate binding proteins range in size from 22-26kdaltons and most commonly exist as trimers or tetramers (Cooper and Barondes, 1981). A lectin of D. discoideum, discoidin I, has been purified and well characterized (Frazier et al., 1975) and molecular biology studies have shown that discoidin I is encoded by a 4-member multigene family (Rowekamp et al. 1980). The discovery of a mutant thought to possess a point mutation in a discoidin I gene and incapable of development beyond the loose aggregate stage seemed to provide strong support for lectins in cell adhesion (Ray et al., 1979; Shinnick and Lerner, 1980). However, in more recent work, discoidin I mutants with known regulatory gene defects showed normal developmental morphogenesis (Alexander et al., 1983). A more recent report (Springer et al., 1984) has shown that discoidin I functions like fibronectin and is needed for streaming and contact with the substratum. In reassessment, the mutant thought to

specific for the active binding site of gp80 or, alternatively, gp80 may not be a specific adhesion molecule but may simply possess the same antigenic determinants as the active adhesion molecules (Murray et al., 1983).

Contact site B has been immunologically identified as a 126kdalton glycoprotein (Chadwick and Garrod, 1983) that functions during vegetative growth in substratum adhesion and as a phagocytic receptor, and during development, in side-to-side cell cohesion and regulation of aggregate size (Chadwick et al., 1984).

In other immunological studies (Parish et al., 1978; Steinemann and Parish, 1980) a glycoprotein of approximately 95kdaltons has been found in slug-stage membranes. In independent studies, Wilcox and Sussman (1981a, 1981b) employed a temperature sensitive mutant that culminated normally at 22°C yet at 27°C formed aggregates which rapidly dispersed to a smooth lawn after 11-12hr of development. Protein separations on SDS polyacrylamide gels revealed that wild-type cells or mutant cells raised at the permissive temperature possessed a 95kd glycoprotein, identified with <sup>125</sup>I wheat germ agglutinin while the mutant at the restrictive temperature lacked this protein. Wilcox and Sussman proposed that the 80kd glycoprotein (contact site A), which functions in cell adhesion during early aggregation, is supplanted or transformed into the 95kd glycoprotein that mediates cell interactions during slug migration and morphogenesis. Recent related work of McConaghy et al., (1984) in the Sussman lab showed that NH<sub>3</sub> and a series of carboxylic acids disrupt cell cohesion in aggregation competent cells; however, these metabolites did not affect cell cohesion after 11-12hr of development. McConaghy et al. speculate that the inhibition of cohesion by these metabolites in early aggregation may relate to the considerable cell reassortment evident in newly formed aggregates (Matsukuma and Durston, 1979; Sternfeld and David, 1981).

during various stages of development, and other carbohydrate binding proteins, similar in many ways to plant and animal lectins, have also been implicated in cell-cell interactions.

#### B Glycoproteins Involved in Cell Adhesion

The majority of potential cell surface molecular signals that have been identified are glycoproteins that function in cell adhesion. Many of these glycoprotein contact sites are developmentally regulated and several cell-type specific adhesion molecules have been reported.

Initial cell adhesion studies (Gerisch, 1961) employed whole Dictyostelium cells in a quantitative assay and determined that one group of contact sites, termed contact sites B, were operative in side-to-side cell adhesions in both vegetative and aggregation competent amoebae. They found that cell adhesions mediated by contact sites B were disrupted by 10mM EDTA. A second group of sites, termed contact sites A, were developmentally regulated (appearing at 10-12hr of development) and these sites which mediated end-to-end cell adhesions were resistant to treatment with EDTA.

Through immunological aggregation-inhibition studies, the adhesion activities of these two sites have been correlated with individual glycoproteins. Contact sites A mediate cell adhesion in aggregation competent cells via a 80kdalton glycoprotein (Beug et al., 1970, 1973a,b; Gerisch 1980; Muller and Gerisch, 1978; Murray et al., 1981). In another study, Murray et al. (1983) were unable to block intercellular adhesion with a monoclonal antibody raised against purified gp80; however, the monoclonal, when bound to an affinity column, did specifically remove gp80 from cell extracts. This affinity purified gp80 successfully neutralized the adhesion-blocking effect of a polyclonal rabbit antiserum to gp80. The monoclonal may not have been

gene expression during in vivo cell differentiation.

Spore cell differentiation of sporogenous mutants of V12M2 was initially found to be density-dependent (Kay et al., 1978; Kay et al., 1979); however, with an improved culture method, spore formation by sporogenous mutants has been found to be density-independent (Kay and Trevan, 1981; Gross et al., 1981). In contrast, Weeks (1982) found that another sporogenous mutant of V12M2, strain Hm18, is density dependent for spore differentiation and has found that plasma membranes can induce spore differentiation in low density monolayers of this mutant. A more recent study (Weeks, 1984) has shown that low molecular weight membrane extracts or conditioned media can induce spore development in low-density monolayers of Hm18. In discussing the variable density requirements for sporogenous mutant differentiation, Weeks suggests that the density-independent mutants used by Kay and coworkers may have less stringent requirements for spore formation and may overcome the requirement for close cell proximity or cell contact by releasing large amounts of a spore-inducing factor into the medium. Earlier reports support the need for a low molecular weight factor in spore differentiation (Wilcox and Sussman, 1978; Sternfeld and David, 1979); and therefore, as suggested above for DIF, close cell proximity and not direct cell contact may allow accumulation of inducible concentrations of this low-molecular-weight factor.

It is interesting to speculate that this low-molecular-weight factor and SPIF are the same signal molecule. The spore coat protein inducing factor (SPIF) described by Wilkinson et al. (1985) is only active if the integrity of the aggregate is maintained. This would suggest that cell contact or at least close cell-cell interactions and the inducing factor are required for spore protein gene expression.

Two general classes of potential cohesion molecules have been studied. Several cell surface glycoproteins may mediate cell adhesion

Gregg, 1971; Landfear and Lodish, 1980; Town et al., 1976; Marin, 1977; Wilcox and Sussman, 1978; Ishida, 1980; West et al., 1983).

Other studies by Kay and coworkers have revised this concept by showing that in vitro cell differentiation of both cell types can occur without cell contact (Kay and Trevan, 1981; Gross et al., 1981; Kay, 1982). As mentioned previously (section 1.2.2C), stalk cell formation occurs in monolayers of the wild-type strain V12M2 in the presence of high concentrations of exogenous cAMP. The formation of stalk cells is density-dependent, suggesting a requirement for cell-cell interactions, but the density dependence can be circumvented if cells are plated at low density in the presence of DIF and high cAMP. In the absence of DIF, close cell interactions may be necessary in order to produce sufficiently high localized concentrations of DIF. Consequently, close cell proximity and not cell contact may be sufficient for in vivo stalk cell differentiation.

As suggested by the need for exogenous cAMP during cell differentiation, DIF alone may not effectively overcome the need for close cell interactions. Landfear and Lodish (1980) have shown that isolated tipped-aggregate stage cells in shaker culture only continue to synthesize stage specific proteins and messenger RNAs if exogenous cAMP is present. They found that if aggregating cells were removed from a solid substratum and resuspended in shaker culture prior to hemispherical aggregate formation, late developmental proteins were not synthesized, even in the presence of exogenous cAMP. They suggest that some developmental event that accompanies formation of tight cell-cell contacts is required for expression of most late genes during in vivo cell differentiation, even in the presence of cAMP. Therefore, close cell interactions or cell contact may be necessary to maintain the levels of intracellular cAMP needed to induce and maintain developmental

and David, 1979) and of mutant FR17 cells (Wilcox and Sussman, 1978) support the view that spore cell differentiation requires soluble factors in addition to cAMP. Kay (1982) has shown in sporogenous mutants of V12M2 incubated at low density that cAMP is the only inducer required for spore cell differentiation. However, this same study found that the wild-type variant V12M2 held at low cell density required other factors secreted by differentiating cells in order to develop mature spores.

In addition to SPIF, Wilkinson et al. (1985) found that the continued expression of the spore coat protein genes also required that integrity of the slug aggregate be maintained; this requirement was not circumvented by adding exogenous cAMP. Therefore, as suggested by many workers (reviewed below), spore-cell differentiation may require multicellularity and continuous cell-cell contacts.

### 1.2.3 Cell-Cell Contact and Cell Differentiation

Cell contact interactions are another mechanism of cell-cell communication in cellular slime moulds. Many cell adhesion molecules have been identified. These molecules in addition to serving as adhesion determinants may also function as membrane bound signal molecules capable of transmitting a specific message and eliciting developmental responses in receiving cells and perhaps also the source cell.

#### A Cell Contact and Cytodifferentiation

Whether cell-cell contact is essential for cytodifferentiation and morphogenesis is yet an open question. Observations of species-specific segregation and morphogenetic movements of prestalk and prespore cells, although consistent with chemotactic mechanisms, also suggest the possibility of selective cell-cell adhesion (Matsukuma and Durston, 1979). Much evidence suggests that cell-cell contact is essential for cell differentiation in cellular slime moulds (Newell et al., 1971;

inhibits a very early developmental event (4-6hr) and this early inhibition then affects later DIF accumulation and stalk-cell differentiation. There may be a correlation between this work and the effect of  $\text{NH}_3$  on cAMP synthesis and secretion, since the cAMP signalling event that initiates aggregation occurs at 4-6hr of development.

#### E Other Potential Morphogens

Other potential differentiation-inducing signal molecules such as SPIF, STF, arachidonic acid, and a low molecular weight factor that disrupts cell cohesion, have been described (Fisher et al., 1981; Dohrman et al., 1984; Hedberg and Soll, 1984; Wilkinson et al., 1985); however, the characterization of most of these factors is quite preliminary. Two of these potential morphogens have been described in some detail and are worthy of mention.

One such factor, slug turning factor (STF), is a low molecular weight (< 500 daltons) factor which disorients the phototaxis and thermotaxis of D. discoideum slugs when tested at high uniform concentration (Fisher et al., 1981). STF is produced by developing aggregates and is a hydrophobic molecule soluble in organic solvents. It has been suggested that cAMP and STF interact as an activator/inhibitor system that specifies cell and aggregate or slug polarity (Meinhardt, 1983; Williams, 1982; Fisher et al., 1984). Collaborative work has verified that STF and DIF, though chemically similar, perform distinct functions in morphogenesis (Williams, 1982).

The second potential morphogen recently reported is SPIF, spore coat protein inducing factor (Wilkinson et al., 1985) and provides another variable in the yet unclear story of spore cell differentiation. SPIF is a low molecular weight ( $M_r$  approx. 100), heat-stable factor that is required for synthesis of six spore coat proteins. Studies of cell differentiation in submerged clumps of wild-type NC4 cells (Sternfeld

acids, cAMP secretion would be reduced. The cells in the centre of the aggregate (or slug) would be "low" cAMP cells since both  $\text{NH}_3$  and acids would be elevated and both cAMP production and release would be inhibited. To continue the interpretation, the "high" cAMP cells, the tip cells, could retain autogenous signalling capacity, even though secretion might be reduced, and would remain chemotactically dominant and guide morphogenetic movements within the aggregate. Williams et al. (1984) have indicated that if the alternative programs of gene expression in the two cell types are regulated by intracellular cAMP levels, then "low" cAMP cells may develop into spores and "high" cAMP cells into stalk/basal disc cells.

This morphogenetic and cytodifferentiation model fits the proposed interactive model for DIF and  $\text{NH}_3$  (Gross et al., 1983). If DIF reduces intracellular pH, as proposed, then DIF would counter the effect of  $\text{NH}_3$  by converting this molecule to  $\text{NH}_4^+$  and only the accumulating acid would affect cAMP accumulation, thus "high" cAMP cells directed toward stalk formation would predominate. Alternatively,  $\text{NH}_4\text{Cl}$  added at high pH would produce "low" cAMP cells and direct cells down the spore pathway. Gross et al. have found that this does indeed occur, therefore, it may not be significant that both cell types can be induced in the presence of high exogenous cAMP levels. The functioning status of the pulsatile, internal cAMP signalling mechanism (the adenylate cyclase system) may be the governing factor in cell type differentiation and intracellular  $\text{NH}_3$  may regulate this signalling mechanism. Another significant point is that if the high  $\text{NH}_3$  levels dictate spore cell differentiation, then spore cell differentiation in vivo may well be cell density dependent, since any disruption of the sheath encased aggregates would allow  $\text{NH}_3$  diffusion.

Other work in the Weeks lab (Neave et al., 1983) reports that  $\text{NH}_3$

formation. However, Morrissey (1982) has pointed out that it is unlikely that ammonia exerts its effect through blocking cAMP production (Schindler and Sussman, 1977) since cAMP probably does not induce pathway specific development (previous section). An alternative role for ammonia is that it may function as an inhibitory morphogen in concert with the putative stalk cell-specific morphogen, DIF (Gross et al., 1981; Gross et al., 1983; Kopachik et al., 1985).

Recent work of Sussman and coworkers (Williams et al., 1984) counters the interpretation of Morrissey and further supports the interactive role of  $\text{NH}_3$  and DIF proposed by Gross. Williams et al. have found that the natural metabolites of amino acid catabolism,  $\text{NH}_3$ , propionate, succinate, pyruvate, and acetate disrupt, in a reversible-manner, cAMP induced cAMP secretion (cAMP relay). Ammonia appears to function by preventing intracellular cAMP accumulation; whereas, the series of carboxylic acids allow intracellular cAMP accumulation but block cAMP release. Williams et al. (1984) propose that these differential effects on cAMP production and secretion could induce cytodifferentiation in the developing aggregate as a consequence of altered intracellular cAMP concentrations. They contend that in a developing aggregate under starvation conditions all cells would be metabolizing amino acids since that is the primary energy and carbon source available (Sussman and Sussman, 1967). As suggested in an earlier report, if the slime sheath surrounding developing aggregates is a minimal thickness at the aggregate apex (Farnsworth and Loomis, 1974), then  $\text{NH}_3$  would more easily diffuse from this area of the aggregate as well as from the base of the aggregate. Microenvironments would then develop where the tip cells possess high acid levels but low  $\text{NH}_3$  and interior aggregate cells would possess both high  $\text{NH}_3$  and high acid levels. Applying the cAMP relay results (Williams et al., 1984), the apex cells would be "high" cAMP cells since, in the presence of only

cell density-independent manner (Brookman et al., 1982). More recent work with purified DIF (Kay and Jermyn, 1983) and mutants defective in the ability to produce DIF (Kopachik et al., 1983, 1985) has confirmed that DIF induces stalk-cell differentiation. The DIF-defective mutant develops only to the hemispherical mound stage; these mutants do not form tips and they lack the prestalk specific isoenzyme of acid-phosphatase (Kopachik et al., 1983). This finding suggests that DIF is necessary for prestalk (as well as stalk), but not prespore, cell differentiation, and further verifies the link between prestalk differentiation and tip formation (Morrissey, 1982).

#### D Ammonia

Ammonia accumulates to significant levels during development and has been shown to have profound effects on the transition from the migrating stage to culmination (Schindler and Sussman, 1977). Sussman and coworkers have proposed that ammonia and cAMP act antagonistically to control morphogenetic movements after aggregation and have suggested that the spatial distribution of cAMP and ammonia in the slug may control the choice between cells entering the prespore or prestalk pathway (Sussman et al., 1977; Sussman and Schindler, 1978; Sussman, 1982). Sternfeld and David (1979), while studying submerged cell aggregates, found that ammonia and another factor from conditioned media was required for differentiation, but, ammonia induced the formation of both spore and stalk cells. Other workers (Gross et al., 1981) have used improved in vitro conditions and found that 2-5mM ammonia dramatically shifts the spore:stalk ratio of sporogenous mutants in favour of spore differentiation. Studies with the "slugger" mutants (Sussman et al., 1978), a short prestalk zone pattern mutant (MacWilliams, 1982), and a cell-contact mutant (Wilcox and Sussman, 1978) all suggest that ammonia may be required for prespore and spore

Cyclic AMP is present both intra- and extra-cellularly throughout development (Bonner et al., 1969; Malkinson and Ashworth, 1972) and it has been suggested that cAMP may induce cells to enter the prestalk/stalk pathway of development (Bonner, 1970; Sussman, 1982; Williams et al., 1984). Brenner (1977) showed that the anterior third of migrating slugs contains a concentration of cAMP approximately 50% higher than the posterior and it has been suggested that a gradient in cAMP concentration in slugs is responsible for the pattern of differentiation and cell fate (McMahon, 1973). High concentrations of cAMP can cause isolated wild-type amoebae to form a small number of stalk cells (Bonner, 1970), or amoebae of a susceptible variant wild-type (strain V12M2) at high cell density to form essentially 100% stalk cells (Town et al., 1976).

However, later studies with mutants from V12M2 indicate that cAMP potentiates prespore and spore differentiation as well as stalk cell formation (Town et al., 1976; Kay et al., 1978). Other studies with a nonhydrolyzable cAMP analogue (cAMP-S) (Rossier et al., 1979), and cAMP soaked Sephadex beads (Feit et al., 1978) further suggest that cAMP induces both stalk and spore differentiation and is therefore not pathway specific. Irrespective of this particular role, cAMP certainly serves many functions as a signal molecule during D. discoideum development (Morrissey, 1982; Sussman, 1982, for reviews).

#### C DIF: Differentiation Inducing Factor

A dialyzable, hydrophobic factor, termed differentiation inducing factor (DIF) is produced by high density developing cells (Town and Stanford, 1979). In vitro stalk cell differentiation in the strain V12M2 (D. discoideum) and sporogenous mutants of this strain is cell density dependent; however, added DIF allows isolated cells of these strains in the presence of cAMP to differentiate into stalk cells in a

later Bonner and coworkers established that this bacterial factor was folic acid (Pan et al., 1972, 1975). These workers proposed that slime mould chemotaxis toward folate had perhaps evolved as a food seeking mechanism. However, several workers have reported that slime mould cells also produce folates and pterins (Pan et al., 1975; Pan and Wurster, 1978; Tatischeff and Klein, 1984) and this may, as others have proposed, suggest a role for such pteridines in later development. Furthermore, pteridines have been established as acrasins in two Dictyostelium species, D. minutum and D. lacteum, where a pterin and a folate derivative have been discovered as their respective species-specific chemoattractants during aggregation (DeWit and Konijn, 1983b; VanHaastert et al., 1982b). These results, which show that folate and pterins are produced by Dictyostelium and function during development, verify that these molecules are important signal molecules in cellular slime moulds.

#### B Cyclic AMP

The significant discovery that cAMP was the acrasin that induced aggregation in D. discoideum, D. mucoroides, and D. purpureum (Konijn et al., 1967; Bonner et al., 1972) has lead to the establishment of many functions for cAMP in slime mould development. Cyclic AMP is produced by an adenylate cyclase enzyme which was originally thought to be localized on the inner face of the plasma membrane (Farnham, 1975); however, more recently, its presence has been reported in vesicles (Hintermann and Parish, 1979). This observation is interesting since apparent fusion of vesicles with the cell surface, occurring in phase with cAMP release, has been described by Maeda and Gerisch (1977). Production and release of cAMP after either folate or cAMP stimulation is the relay response (to be discussed in more detail later in this chapter).

factor (SPIF) (Wilkinson et al., 1985) could also transit the cell membrane and contact internal receptors of the receiving cell (Cell B).

#### E The Response

As in any communication event, the event cannot be considered complete until there is evidence that the message has been received. In human communication, a gesture or verbal response would indicate that a verbal message had been received. In cellular communications, the cellular response can be quite varied. The response can be a morphogenetic response, such as conversion from the migration stage to the fruiting body stage after environmental change. Many cell responses thought to indicate signal transduction after signal/receptor interactions have been studied in cellular slime moulds (for review see Devreotes, 1982; and section 1.2.5). In the sections that follow, the possible messages that may induce these cellular responses are discussed in more detail, and separately, the role of cell-cell contact in D. discoideum is reviewed. The role of receptors is not further introduced; evidence for folate receptors, the receptor of predominate interest in this study, is presented in chapter 3. A review of the many cell responses that may be induced by signal molecules, the environment, or cell-cell contact is also presented.

#### 1.2.2 Signal Molecules as Potential Morphogens

##### A Folates and Pterins

Folates are potent chemoattractants of all species of cellular slime moulds so far tested (Pan et al., 1975; DeWit and Konijn, 1983a). A main source of folates in the natural environments of slime moulds are the soil bacteria. Samuel (1961) and Bonner et al. (1966) described a factor produced by bacteria that attracted cellular slime moulds and

further processing. Alternatively, if the information source is from a particular cell type, then the message would be generated within the cell via gene expression and then presented on the cell surface either as a secreted signal molecule or as a cell-surface signal.

#### C The Medium

The medium of signal transmission for the chemical signal is the extracellular fluid that fills the extracellular space. The signal can function over a large distance if the conditions are favourable and the signal chemical is stable and capable of functioning at low concentrations. In the case of a signal bound to the cell surface, the medium is essentially non-existent since cells must contact directly before the message can be transmitted from the cell-surface molecule on the source cell (Cell A) to a complementary receptor on the recipient cell (Cell B).

#### D The Receptor

The receiving cell (Cell B) must possess some type of signal receptor. In the case of the chemotactic signals folate and cAMP, specific receptors are known to be present on the cell surface (Wurster and Butz, 1980; Malchow and Gerisch, 1974; Green and Newell, 1975). Also, it would be expected that the signals bound to the surface of the source cell (Cell A), would have a complementary cell-cell contact receptor on the surface of the receiving cell (Cell B). It is additionally possible that certain signal molecules can transit the cell membrane and contact internal cell receptors. Molecules or ions such as  $H^+$ ,  $OH^-$ ,  $NH_3$ , and stimuli such as light and heat can easily transit the cell membrane and function by altering the internal environment of the receiving cell. In such cases, the internal receptor may not be well defined. The non-polar, lipid-like differentiation inducing factor (DIF) (Kay et al., 1983) and perhaps the spore coat protein inducing

show normal morphogenesis, actually displayed aberrant streaming characteristics.

If lectins do indeed function in cell-cell adhesions, then one would expect that lectin receptors in the form of glycoproteins are present on the surface of developing slime mould cells, since lectins are carbohydrate binding proteins. Developmentally regulated discoidin I and discoidin II receptors have been demonstrated on glutaraldehyde-fixed, aggregation-competent D. discoideum cells by agglutination of these cells with purified lectins. Agglutination and apparent lectin binding are inhibited by N-acetyl-D-galactose, indicating that agglutination is mediated by lectins with specific carbohydrate binding sites. Many attempts to isolate lectin receptors from cell digests have been complicated by bacterial and growth media glycoproteins that also bind to the discoidin (Barondes et al., 1982). Using affinity columns with covalently linked discoidin I, Breuer and Siu (1981) and Ray and Lerner (1982) have isolate presumptive receptors of 31kdalton and 80kdalton, respectively.

#### 1.2.4 Cell Responses

Many cellular and molecular responses to chemotactic signals are thought to result from transduction of the signal from the cell surface receptors to the intracellular components controlling cell movement. These responses are summarized below.

##### A Light Scattering Response

Stimulation of slime mould cell suspensions by the stage specific chemoattractant (e.g. folate or cAMP) results in a transient decrease in optical density of the suspension and may reflect a responsive change in cell shape or the state of cell aggregation (Gerisch and Hess, 1974). Futrelle et al. (1982) have shown that cells round up or "cringe" after cAMP or folate stimulation and then elongate for chemotaxis; this

behaviour may account for the biphasic light scattering response seen in cell suspensions. Light scattering responses are unaffected by the addition of EDTA, known to disrupt cell-cell contacts, thereby suggesting that shape change is responsible for the decrease in optical density (Wurster et al., 1978).

#### B Increases in cGMP Levels

Rapid and transient increases in intracellular cGMP levels occur after stimulation of cell suspensions with species-specific and developmental stage-specific chemoattractants (Mato et al., 1977; Wurster et al., 1977). This response is reviewed in more detail in Chapter 9.

#### C Calcium Mobilization

Stimulation of D. discoideum amoebae with physiological concentrations of cAMP or folate, or high concentrations of cGMP induces a transient uptake of calcium from the medium within 6sec which peaks by 30sec (Wick et al., 1978; Bumann et al., 1984). Other work suggests that only intracellular calcium stores are essential for signal transduction (Mato et al., 1977; Europe-Finner and Newell, 1984). The recent work of Aeckerle et al. (1985) in the Malchow lab, has shown that cAMP stimulations of aggregation competent cells result in a rapid and transient efflux of  $K^+$ . These workers point out that the  $K^+$  efflux corresponds temporally with  $Ca^{2+}$  influx after stimulation and suggest that  $K^+$  efflux may be activated by  $Ca^{2+}$  influx. The essential role of calcium mobilization in signal transduction is reviewed in more detail in chapter 10.

#### D Changes in Extracellular pH

Decreases in the extracellular pH of unbuffered suspensions of aggregation-competent D. discoideum cells are detected with 45sec after

cAMP stimulation (Malchow et al., 1978a,1978b). It is unknown whether the change is due to excretion of protons, an undissociated acid, or a dissociated weak base.

#### E Protein Methylation

Cyclic AMP stimulation of aggregation competent cells induces a rapid two-fold increase in the methylation of a 120kdalton D. discoideum plasma membrane protein (Mato and Marin-Cao, 1979). The peak of methylation is reached within 15sec and intracellular calcium levels may regulate this response (Mato and Marin Cao, 1979; Nuske, 1980). In a more detailed study, Van Waarde (1982) found that at least four proteins are transiently methylated; one protein of 46kdaltons reached peak levels of methylation within 3sec after cAMP stimulation. He additionally showed that this same protein is methylated in D. discoideum vegetative amoebae after folate stimulation and in D. lacteum following pterin stimulation (Van Waarde, 1983). Several transmethylation inhibitors prevent these protein methylations, partially block chemotaxis, and delay cell aggregation (Van Waarde and Van Haastert, 1984).

#### F Phospholipid Methylation

Originally, demethylation of phospholipids was shown in D. discoideum within 2min after cAMP stimulation (Mato and Marin-Cao, 1979). In contrast, other workers (Alemany et al., 1980) found that cGMP (at a concentration normally found after chemoattractant stimulation) increased the activity of a methyl transferase in a homogenate of D. discoideum cells. This enzyme appeared to mediate methylation of phosphatidyl-ethanolamine to form phosphatidylcholine by catalyzing the transfer of methyl groups from S-adenosyl-L-methionine. In vivo experiments revealed similar effects following cAMP stimulation; the time course of methylation and a similar response with the membrane

permeant 8-bromo-cGMP suggested that cGMP may mediate this response. Methylation of phospholipids appears to be much slower than protein methylation and peaks at about 1min (Alemany et al., 1980).

#### G Protein Phosphorylation

The dephosphorylation of myosin heavy chains may occur after cAMP or folate stimulations (Rahmsdorf et al., 1978; Malchow et al., 1981). In vitro incorporation of  $^{32}\text{P}$  onto myosin heavy chains is inhibited by 0.1mM  $\text{Ca}^{2+}$ ; this effect may be mediated by calmodulin and may result from inhibition of the myosin heavy chain kinase (Malchow et al., 1981; Maruta et al., 1983). Therefore increases in cytosolic  $\text{Ca}^{2+}$  may act via calmodulin to inhibit the kinase resulting in dephosphorylation of myosin heavy chain. This is particularly interesting in light of the report by Kuczmarski and Spudich (1980) that dephosphorylation of myosin heavy chain favours formation of thick filaments and increases the activity of actin-activated myosin ATPase by 80% and may, therefore, regulate contractile events within the cell.

#### H Cyclic AMP Production and Secretion

The cAMP relay response, described briefly during the earlier discussion of cell aggregation (section 1.1.1B), is a transient increase in cAMP production and secretion after cAMP stimulation (Shaffer, 1975; Roos et al., 1975; Gerisch and Wick, 1975; for review, Devreotes, 1982). Aggregation competent cells of D. discoideum also secrete cAMP after stimulation with folate, pterin, xanthopterin, amethopterin, and aminopterin. The maximum level of cAMP formed by NC4 in response to these pteridines is only 5% of that after cAMP stimulation. However, in the strain AX3, the maximal cAMP secretion response induced by folate and xanthopterin are often greater than those elicited by cAMP. This may be due to a much stronger positive feedback loop seen in AX3 as the

cAMP, produced after folate stimulation, further stimulates cAMP production via the cAMP receptor (Devreotes, 1982).

#### I Chemotaxis Response

Vegetative amoebae and aggregation competent amoebae are able to detect gradients of folate and cAMP, respectively, and move in the direction of the higher chemoattractant concentration. The mechanism by which cells are able to detect minute differences in concentration between one side of the cell and the other is unclear. The two extreme views are: 1) Cells detect concentration differences in time by sampling the environment at different times and at different points in the gradient as they move through it or as it moves past them. This is the temporal sensing mechanism so well characterized in bacteria (Hazelbauer, 1981). 2) Cells sense a spatial gradient over their surface by sampling their surroundings at different points but at the same time. Such a spatial sensing mechanism has been suggested for amoeboid cells such as leukocytes (Zigmond, 1978). Many mechanisms have been proposed to explain how Dictyostelium amoebae are able to orientate themselves in a chemotactic gradient but no single mechanism of choice predominates (Devreotes, 1982).

#### J Actin Response

Chemotactic stimulation of several slime mould species with their species specific chemoattractant causes a series of rapid changes in the amount of actin protein associated with the Triton X-100 insoluble cytoskeleton (McRobbie and Newell, 1983, 1984a). The first of these changes occurs within 5sec in all species tested and corresponds temporally with pseudopodium formation. Similar responses have been characterized in leukocytes (White et al., 1982, 1983; Rao and Varani, 1982), and in thrombin stimulated platelets (Carlsson et al., 1979; Jennings et al., 1981). In the case of D. discoideum, the rapid actin

increase is followed by two further peaks of actin association with isolated cytoskeletons; these other changes may correlate with other cell movements and shape changes (McRobbie and Newell, 1983). McRobbie and Newell (1985a) found that the most acidic isoform of three major isoactins preferentially accumulates in the cytoskeleton in response to chemotactic pulses. The accumulation of actin after stimulation apparently involves actin polymerization (McRobbie and Newell, 1985b). The actin-filament capping drug, cytochalasin B, known to inhibit actin polymerization, abolishes the second and third peaks of actin accumulation but does not affect the 5sec peak. Further studies, employing a DNase I inhibition assay, showed that the 5sec peak may also involve actin polymerization since a rapid reduction in cytosolic G-actin occurs after ligand stimulation, suggesting a shift to the filamentous form.

### 1.3 MODELS TO EXPLAIN CELL PROPORTIONING AND PATTERN FORMATION

In recent years, a number of models have been introduced to explain cytodifferentiation and pattern formation in D. discoideum (for reviews, MacWilliams and Bonner, 1979; Morrissey, 1982). The majority of these models are based on events that have been observed within the migrating slug, but the more tenable models also accommodate the evidence of cytodifferentiation in the tipped aggregate. As suggested by Morrissey (1982), the various models can be divided into two categories. The first group of models are those in which cell position within the aggregate or migrating slug dictates the developmental pathway followed by the cell. In the second category, the cell types are proposed to develop at random within the developing aggregate in a manner that establishes cell proportions; the final pattern is then established

through a sorting out process.

The models that propose the position-dependent differentiation of a homogeneous population of cells into prestalk and prespore cells and ultimately a consistent proportion of stalk and spore cells generally suggest that chemical gradients are established within the aggregate which provide the positional information to the cells. The Sussman model presented in some detail while discussing ammonia as a potential message (section 1.2.2 D) suggests that the chemical gradient develops through differential diffusion through the slug sheath. This example proposes that the gradient of ammonia provides the position information and dictates cell fate (Sussman and Schindler, 1978; Sussman, 1982; Williams et al., 1984). The theory of Kay and colleagues suggests that DIF and ammonia coordinately induce stalk and spore differentiation, respectively, in a position-dependent manner. The DIF/ammonia scheme fits the activator-inhibitor model of cell differentiation (Gierer and Meinhardt, 1972) which has been adapted to pattern formation in D. discoideum slugs (MacWilliams and Bonner, 1979; Meinhardt, 1983). In this activator-inhibitor model, an activator substance not only stimulates its own synthesis but also indirectly inhibits its own production by stimulating the production of an inhibitor. By varying the rates of diffusion and breakdown (both substances spontaneously decay), the inhibitor is made to have a longer diffusion range than the activator.

The models that involve position-dependent cell differentiation do not account for the apparently random distribution of prestalk and prespore cells initially seen in the hemispherical and tipped aggregate during development (Forman and Garrod, 1977; Tasaka and Takeuchi, 1981). These observations lead to the proposed models that involve random cell differentiation and then cell sorting. Any such model must account for regulation of cell proportions during random cell differentiation and

then sorting out of cell-types to establish the observed slug pattern. At the expense of reducing our perspective, two models will be presented as examples. Durston and Weijer (1980) proposed that each cell type secretes an inhibitor of its own type of differentiation; that is prestalk cells secrete a substance that blocks prespore to prestalk conversion and vice versa. The mathematical analysis of this model shows, surprisingly, that cell proportioning can be established without spatial localization of the morphogenetically active substances.

Alternatively, the Meinhardt (1983) model of position-independent random cell differentiation is an altered version of the activator-inhibitor scheme and requires localized and differential rates of diffusion of an activator and a depleted-substrate. The depleted substrate version of the model was developed from analysis of the stalk cell-inducing roles for DIF described by Kay and coworkers. Meinhardt reasoned that, if the scheme involved an inhibitor, then exposure of cells at low density to diffusable components from the medium of cells developed at high cell density would reduce the appearance of one cell type; whereas if the diffusable substance was a substrate then additional amounts of the factor would result in increased differentiation of a particular cell type. Since DIF preferentially increases the proportions of stalk cells in low density monolayers, Meinhardt favoured the activator-depleted substrate model. In either this model or the Durston and Weijer idea, following cell differentiation in the proper proportions, there must be a functional mechanism of establishing the pattern of isolated prespore and prestalk cells.

Morrissey (1982) presents three mechanisms by which the segregated cell pattern may be established: 1) differential rates of migration of the two cell types, 2) chemotaxis of prestalk cells towards each other,

and 3) differential adhesion. The first possibility seems least likely since cell sorting is observed in aggregates in liquid cultures where aggregate migration cannot occur (Forman and Garrod, 1977; Tasaka and Takeuchi, 1981). Meinhardt (1983) has shown preference to a chemotaxis mechanism that includes pulsatile signaling from the tip cells. However, the differential adhesion concept also has merit. Several workers have observed cell sorting in shaker culture (Forman and Garrod, 1977; Tasaka and Takeuchi, 1981) and have suggested that cell adhesion must be the mechanism since functional chemotactic gradients are unlikely in liquid, shaking cultures. The work of Lam et al. (1981), which showed that antibodies against the glycoprotein gp150 were more effective in dissociating prespore cells than prestalk cells, may also support a role for cell cohesion in cell sorting. Other researchers (Matsukuma and Durston, 1979) have supposed that both chemotaxis and cell adhesion could function in pattern development.

#### 1.4 SIGNAL TRANSDUCTION IN DICTYOSTELIUM

Biochemists are rapidly beginning to discover and understand the intricate mechanisms involved in transduction of externally generated signals that lead to a wide variety of internal responses. In Dictyostelium, the wide spectrum of signal molecules and other potential environmental messages (section 1.2.3) seem to generate a rather diverse yet common series of cellular responses (section 1.2.5). Several models designed to explain transduction of external messages in Dictyostelium discoideum have been presented. DeWit and Konijn (1983a) have presented one model which attempts to incorporate the aspects of cGMP accumulation, cAMP relay, observed adaptation to increases in signal concentrations, and the apparent convergence of the transduction pathway after signal initiation at a variety of cell surface receptors (figure 1.3a). Van Haastert (1983) was unable to demonstrate an additive

Figure 1.3      Signal Transduction in Dictyostelium  
and Higher Eukaryotes

a) McRobbie and Newell model of signal  
transduction in Dictyostelium  
(McRobbie and Newell, 1984).

b) DeWit and Konijn model of signal  
transduction in Dictyostelium  
(DeWit and Konijn, 1983).

c) Inositol triphosphate and  
diacylglycerol ( $IP_3$ /DG) mediated signal  
transduction (Berridge, 1984).

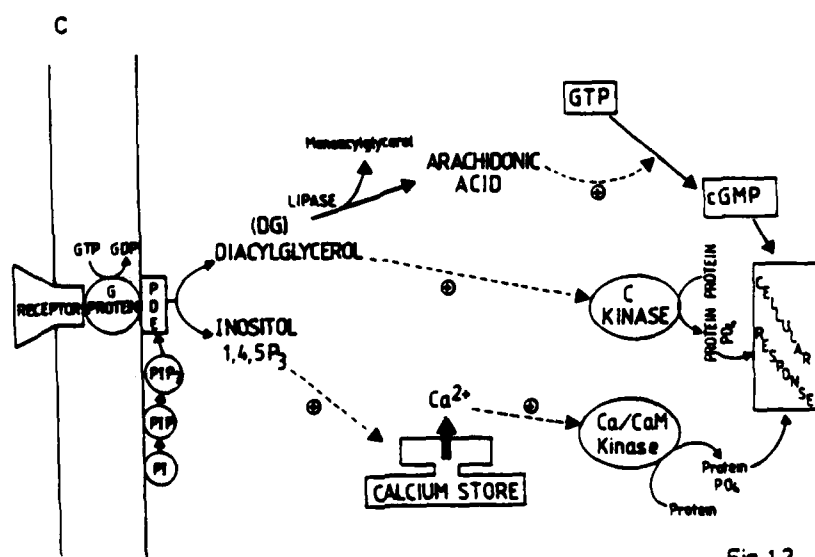
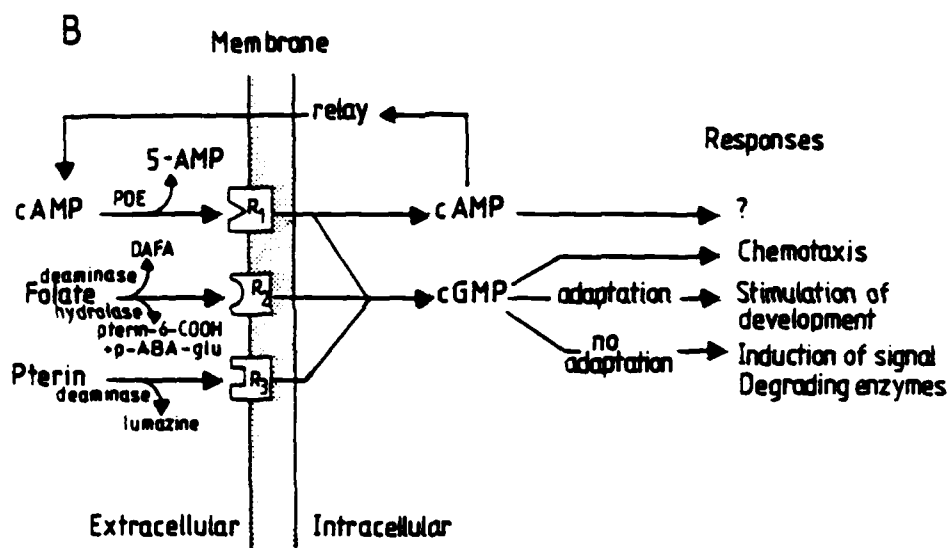
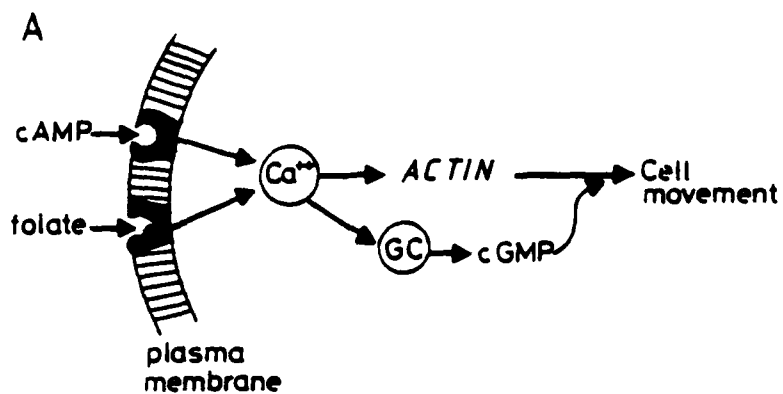


Fig 13

cGMP effect when cells were stimulated simultaneously with both cAMP and folate; he, therefore, proposed the common, convergent pathway for guanylate cyclase activation. In recent studies, the Konijn lab has continued to present evidence which may explain the mechanism of convergent transduction from diverse signals. The recent reports of GTP binding proteins that may mediate signal transduction after either cAMP or folate stimulation may further explain this process of signal convergence (Leichtling et al., 1981; VanHaastert, 1984; DeWit and Bulgakov, 1985).

McRobbie and Newell (1984b) have presented an alternative signal transduction scheme for Dictyostelium which accomodates the divergent roles for  $\text{Ca}^{2+}$  and cGMP, observed in the ligand-induced actin response (figure 1.3b). McRobbie and Newell (1984b) demonstrated an additive effect in the actin response after stimulations with cAMP and folate. Therefore, they modified the signal transduction model to include distinct pathways for folate and cAMP stimulation of the actin response. From other evidence they showed a third pathway for guanylate cyclase activation, and postulated a common role for  $\text{Ca}^{2+}$  in all pathways.

In comparison to the rather simple pathways for signal transduction so far evident in Dictyostelium, models for signal transduction in other eukaryotic systems are distinctly more complex (figure 1.3c)( for review, Berridge, 1984). It is important to note, however, that these more descriptive models still include cGMP and  $\text{Ca}^{2+}$  as central components.

These three models have been introduced here for comparison and will be discussed in more detail later, in the context of experimental results.

### 1.5 Aim of This Study

The actin response described by McRobbie and Newell (1983) provides a new method for assessing cell responsiveness to ligand-signals. The indication that folate induces an actin-related chemotactic response at 12hr of development suggests an alternative role for folate beyond the vegetative stage (where folate functions in the food seeking mechanism). I, therefore, chose to investigate further the role of folate in the tipped aggregate (12hr) stage of development.

I established the following working hypothesis: Folate serves as a chemotactic signal in tipped aggregate formation and functions to induce morphogenetic development.

If folate does function as a signal molecule in morphogenetic development or cytodifferentiation, I reasoned that it should be possible to demonstrate the presence of components of a communication system (introduced in section 1.2) in developing aggregates where folate functions as the signal molecule. From the review of previous literature (section 1.2.2a) it is evident that folates and pterins are produced by developing Dictyostelium cells and therefore a source of potential signal molecules exists.

The aim of this study was to identify other components of the folate mediated communication mechanism in the developing aggregate. The approach was: first, to demonstrate the presence of folate receptors in wild-type cells at the tipped aggregate stage of development and to characterize the specificity of these receptors; secondly, to demonstrate that folate did function in cell-cell communication during tipped aggregate formation by studying folate-induced cellular responses in cells from this developmental stage; and additionally, to further understand the role of cell-cell contact interactions in tipped aggregate formation by studying a series of developmental mutants that were unable to form tipped aggregates.

## *Chapter 2*

Cells were fixed by immersing the slide in 100% methanol, 4°C for 1.25min followed immediately by three-5min soaks in PBS. Smears were allowed to dry briefly (1-2min) and then incubated with 10µl anti-PV serum for 30min at room temperature in a humidified chamber. This was followed by three-5min PBS soaks and then incubation with tetramethylrhodamine isothiocyanate (TRITC) conjugated sheep anti-rabbit serum (10µl per field, room temperature, humidified chamber). Finally, the smears were again washed by three-5min soaks in PBS, dried briefly, mounted under a coverslip in glycerol:PBS (9:1) and sealed around the coverslip with nail varnish. Fluorescence was observed using a Leitz microscope equipped for epifluorescence microscopy.

#### L Chemotaxis Assays

Chemotactic competence of cells was determined by two methods, either a "spreading" assay discussed by Bonner et al. (1966, 1970) and more recently by workers in the Soll lab (Finney et al., 1979; Varnum and Soll, 1981) or a "directional" assay employed in several labs (Finney et al., 1979; Nandini-Kishore and Frazier, 1981) and modified during this study.

In the "spreading" assay, cells in a high density spot were tested for their ability to detect a chemoattractant in the agar substratum and then move outward from their original position. Bonner et al. (1966, 1970) have discussed the basis of this assay. Briefly, it depends on the cell production and secretion of enzymes (phosphodiesterase or folate deaminase) which specifically degrade the attractants (cAMP, folate) in the surrounding agar. This degradation creates a gradient of attractant around each spot and chemotactically competent cells will respond by moving up such a gradient, outward from the original cell spot, in a "spreading" fashion.

The "spreading" assay was conducted in 90mm petri dishes containing

and the mean weight determined. Quantitative changes in a particular protein as a result of chemotactic stimulation could be calculated by comparing the scans from different samples. Such differences were expressed as a percentage change in protein content and were plotted graphically versus the time following stimulation.

Total Protein Determinations. Total protein determinations of whole cell preparations employed the Bio-Rad protein assay reagent (Bradford, 1976) using bovine- $\gamma$ -globulin as standard. Samples in SDS were diluted sufficiently ( $<0.1\%$ ) such that the SDS did not interfere with readings.

#### K Immunofluorescence Labelling of Prespore Vesicles

Prespore vesicles were identified as a prespore cell marker by the method of Takeuchi (1963) with modifications suggested by others (Johnstone and Thorpe, 1982). The prespore vesicle antiserum (anti-PV) was produced by Dr. James Morrissey following the method of Takeuchi (1963).

Microscope slides with eight circular etched fields (1/4 inch diameter) were cleaned thoroughly in acetone and ethanol and then spotted in the etched fields with 10  $\mu$ l aliquots of poly-L-lysine, 0.1 mg  $\text{ml}^{-1}$  in deionized water. After 1 hr in a humidified chamber at room temperature, the slides were rinsed in deionized water and then allowed to dry after wiping excess moisture from around the fields.

Cells to be tested were suspended at approximately  $10^6$  cells  $\text{ml}^{-1}$  in phosphate buffered saline (PBS, 10 mM  $\text{Na}_2\text{K}$ , 0.14 M NaCl, 1 mg  $\text{ml}^{-1}$  sodium azide, pH 7.0), then kept on ice to reduce dedifferentiation. Cell suspensions were spotted in 8  $\mu$ l aliquots to the dried poly-L-lysine-coated fields and allowed to settle and attach during 30 min at  $4^\circ\text{C}$  in a humidified chamber. Excess buffer was aspirated from the centre of the fields with a drawn-out pasteur pipette and smears allowed to dry briefly (5 min) while drying excess buffer from around the fields.

treated were destained in 20%(v/v) methanol, 10%(v/v) glacial acetic acid overnight in the presence of foam bungs to absorb released stain. Gels were stored in 10%(v/v) acetic acid.

The more sensitive method was the silver staining technique of Morrissey (1981). First, the gels were fixed in 50%(v/v) methanol, 10%(v/v) acetic acid for 30min then in 5%(v/v) methanol, 7%(v/v) acetic acid for an additional 30min. Fixation was continued for another 30min in 10%(v/v) glutaraldehyde. The gels were then rinsed overnight in several changes of deionized water to remove all traces of fixative and then soaked for 30min in  $5\mu\text{g ml}^{-1}$  DTT for 30min. After pouring off the DTT solution, the gels were soaked for 30min in 0.1%(w/v) silver nitrate. The silver nitrate solution was removed and the gels rinsed quickly once with deionized water and twice with a small amount of developer (50 $\mu\text{l}$  of 37%(v/v) formaldehyde in 100ml of 3%(w/v)  $\text{Na}_2\text{CO}_3$ ) and resuspended in additional developer. While being observed over a light box, the gels were stained until sufficient intensity was attained. Staining was stopped by adding 5ml (per gel) 2.3M citric acid and agitating for 10min. Gels were then washed for 30min in several changes of deionized water and finally stored in 0.03%(w/v) sodium carbonate to prevent bleaching. Gels were heat sealed in commercial plastic freezer bags in 0.03%(w/v) sodium carbonate when retained for future reference.

All volumes of reagents used were 100ml per 9cm x 13cm x 1mm gel except for the glutaraldehyde which was 50ml.

## J Quantitation of Proteins

Scanning Densitometry. A Joyce Loebel double-beam recording microdensitometer MkIII C8 was used to scan the protein bands of interest in one-dimensional gels. Each band was scanned across its width five times, peaks from the resulting recorder traces were cut out

room for the sample.

Once the gels polymerized, the bottom section of the tubing containing sealing compound was broken off and the tubes were then placed in a tube-gel apparatus modified to accommodate the smaller diameter tubing. The lower (anode) electrode chamber was filled with 0.01M  $H_3PO_4$  and the upper (cathode) electrode chamber with 0.01M NaOH, 0.01M CaO. Using a microsyringe each gel was overlaid with 3 $\mu$ l of an 8M urea solution containing 8M urea, 4%(w/w) NP-40, 10mM DTT, 2%(w/v) pH 5-7 ampholytes made up in water. The samples were loaded immediately, since for NEPHGE no pre-focusing of the ampholytes is required (O'Farrell et al., 1977). Care was taken to load the sample (10M urea) beneath the overlay (8M urea). These gels were run at 1000V for 2 hr.

On completion of first dimension electrophoresis, the tubes were removed from the apparatus and cooled with dry-ice at intervals along the tube to precipitate the urea and then stored temporarily on ice. Using a compressed air source, gels were cautiously blown from the tubes into an equilibration solution composed of 62.5mM Tris-HCl, pH6.8, 10%(w/v) SDS and 50mM DTT. Equilibration was reached in 90sec and the gels were then separated from the equilibration solution and placed directly into the upper electrode reservoir of the slab-gel apparatus. Gels were teased into place along the top of the slab gel with aid of small spatulas.

Second-dimension separation was completed in 9cm deep polyacrylamide gels as described in the preceding section.

It is important to note that all gel solutions were filtered whenever possible to remove dust etc., which has been proposed to interfere with silver staining processes (Morrissey, 1981; Ochs, 1983).

#### I Staining of Proteins in Polyacrylamide Gels

Proteins were stained by one of two methods, the first being Coomassie staining in 50%(w/v) brilliant blue R for 2.5hr. Gels so

samples were stored at  $-70^{\circ}\text{C}$ .

#### G SDS-Polyacrylamide Gel Electrophoresis

Electrophoresis was conducted in 1mm thick, 13cm x 9cm slabs of 10%(w/v) polyacrylamide. Samples were loaded using a microsyringe in 1cm wide wells formed in a 4cm deep stacking gel of 4.5%(w/v) polyacrylamide. Polymerization was achieved using the ammonium persulphate/TEMED system.

The gel buffers were of the following compositions: separating gel; 1.5M Tris-HCl pH8.8, 0.4%(w/v) SDS. stacking gel; 0.5M Tris-HCl pH6.8, 0.4%(w/v) SDS.

Gels were run on a Studier-type apparatus (Studier, 1973) according to the method of Laemmli (1970) at a constant current of 15mA per gel until the tracking dye (bromphenol, 0.005%(w/v), added as a few drops to the upper electrode chamber) reached the bottom of the gel, approximately 4 1/2hr after beginning electrophoresis. Electrode buffer contained 25mM Trizma base, 192mM glycine and 0.1%(w/v) SDS, yielding an approximate pH of 8.3.

#### H Two-dimensional Polyacrylamide Gel Electrophoresis

First-dimension separations were by non-equilibrium pH gradient electrophoresis (NEPHGE) (O'Farrell et al., 1977) as modified by Devine et al. (1982).

These gels were cast in 13cm lengths of glass tubing of 1.0-1.2mm internal diameter, by filling the tubes, through capillary action, with a solution of the following composition: 2.4%(w/v) polyacrylamide, 10M urea, 4%(w/w) NP-40, with 2%(w/v) ampholytes of the pH range 3.5-10. Only ammonium persulphate was required to polymerize these gels. The filled tubes were sealed at one end with Cristaseal sealing compound and the top 2cm of polyacrylamide solution was removed by a syringe to allow

The 2x Triton stock solution contained 2% (v/v) Triton X-100 (electrophoresis grade), 20mM potassium chloride, 20mM imidazole, 20mM EGTA and 4mM sodium azide (pH7.0).

#### F Sample Preparation for Gel Electrophoresis

One-dimensional SDS-PAGE. Protein pellets were dissolved by heating to 80°C for 10min in sample buffer containing 62.5mM Tris-HCl (pH6.8), 2%(w/v) SDS, 10mM DTT and 10%(v/v) glycerol.

Whole cells were prepared by two methods. In the first, cells were pelleted at 8000xg for 30 sec in a Beckman Microfuge B and the pellet was then treated similarly to the protein pellets. In the second, a cell suspension was treated with an equal volume of 2x sample buffer (containing the components listed above at twice the concentration) and was then heated at 80°C for 10min.

Two-dimensional Gel Electrophoresis. This method was modified from that of Devine et al. (1982) and the reagent volumes quoted are those necessary to give a final sample volume of 100µl.

The protein or whole cell pellet was first suspended in 36µl of 1%(w/v) SDS plus 8µl of 55mM DTT, both in 20mM Tris-HCl (pH7.4), and boiled for 10min. After cooling to room temperature, 8µl of a DNase/RNase mix was added and left for 10min (composition: 20mM Tris-HCl, pH7.4, 32mM MgCl<sub>2</sub>, 0.65mg ml<sup>-1</sup> DNase I, 0.33mg ml<sup>-1</sup> RNaseA). Finally, 5µl of 40%(w/v) ampholytes (pH5-7); 9µl 44%(w/v) NP-40, 62mM DTT in water; and 60mg of urea (giving 10M final concentration) were added, the urea being dissolved at room temperature.

Molecular Weight Standards. The standard mix was prepared by heating to 80°C for 10min in one-dimensional SDS-PAGE sample buffer. The following amounts of protein were present in a typical 10µl sample load; rabbit muscle myosin (205,000M<sub>r</sub>) 6.4µg, B-galactosidase (116,000M<sub>r</sub>) 3.2µg, bovine serum albumin (66,000M<sub>r</sub>) 4.0µg, rabbit muscle actin (43,000M<sub>r</sub>) 3.2µg, and carbonic anhydrase (29,000M<sub>r</sub>) 3.2µg. All

clones appearing on the two types of agar was recorded for each population ratio.

#### E Isolation of Cytoskeletal Proteins

Cytoskeletal proteins were isolated as proteins insoluble in Triton X-100 (Brown et al., 1976; Osborn and Weber, 1977) using a previously published method (Rosenberg et al., 1981; White et al., 1982; McRobbie and Newell, 1983, 1984a).

Cells were harvested from starving shaker suspension at the desired point in development or from Millipore filters, centrifuged at 190xg for 2min and resuspended at  $10^8$  cells ml<sup>-1</sup>. Samples (usually 150µl) were then distributed to 1.5ml Eppendorf microcentrifuge tubes shaken at 1400rpm on a Janke and Kunkel IKA-Vibrax-VXR shaker at room temperature. Chemotactic stimulation was accomplished by adding 10µl of an appropriate chemoattractant to the shaking tubes, allowing the reaction to proceed for a preset time and then terminating the reaction by addition of 150µl of 2X Triton stock solution (stored at 4°C and warmed to room temperature prior to use).

Following termination of the reactions, the tubes were placed on ice for 10min, then allowed to warm to room temperature for 10min with occasional agitation. The Triton-insoluble cytoskeletons were pelleted by centrifugation in a Beckman Microfuge B for 4min at 8000xg. The supernatants were removed and each pellet resuspended in 300µl of 2x Triton stock diluted 1:1 with SORPHOS. The tubes were then recentrifuged, supernatants again removed, and the tubes inverted to dry.

The protein pellet thus obtained is widely termed the cytoskeleton (Brown et al., 1976; Osborn and Weber, 1977; Rosenberg et al., 1981; McRobbie and Newell, 1983, 1984a) and I will routinely refer to it as such.

were induced by incubation for 30-40hr in a dark humidified chamber with a point light source and with the dish slightly inclined toward the drain hole.

Development on Buffered Agar. Clearing mass plates were washed free of bacteria and resuspended at  $3 \times 10^7$  cells  $\text{ml}^{-1}$  in SORPHOS. Samples (1ml) of amoebae were dispensed onto KMMP agar in 50mm petri dishes, allowed to settle for 15-30min, then excess liquid was decanted, and the plates were air dried for about 20min until the layer of amoebae had an even matt appearance. The plates were held at  $7^\circ\text{C}$  for 17hr and then transferred to  $22^\circ\text{C}$  to initiate synchronous development.

#### D Synergistic Development of Mutant and Wild-type Amoebae

Washed cell suspensions from clearing mass plates of both wild-type cells (e.g. NC4) and mutant cells (e.g. NP551) were prepared and mixed in appropriate dilutions to obtain a complete range of population ratios (Sussman, 1955). A frequently used range was from 10% mutant:90% wild-type to 90% mutant:10% wild-type. The mixtures were then placed on either KMMP agar or on Millipore filters over pads and allowed to develop as described above. After 48hr of development, the morphological appearance of the spectrum of chimeras was recorded; and in those mixtures where culmination occurred, the spores were harvested by a sterile wire loop for further analysis.

The spores from each population ratio were suspended in SORPHOS containing 10mM EDTA and 0.1%(v/v) Nonidet P-40 detergent (NP-40). The detergent served to lyse any nonsporulated amoebae (Devine et al., 1982). Spores were then washed once in SORPHOS and diluted sufficiently to obtain  $10^3$ ,  $10^2$ , or  $10^1$  spores per 90mm agar plate. Identical samples of spores and bacteria were plated onto SM agar and SM agar containing a drug to which the wild-type was sensitive (e.g. methanol in the NC4/NP551 synergy experiment). After 24 to 36 hours, the number of

harvesting the clearing plates into SORPHOS and washing the amoebae free of bacteria by centrifugation at 190xg for 2min. After four such washes the cells were resuspended in the same buffer at a density of  $2 \times 10^8$  cells  $\text{ml}^{-1}$  and plated onto 47mm Millipore filters (HAWP or HABP) resting on pre-filter pads (Millipore No. AP1004700) saturated with SORPHOS or lower pad solution (Sussman and Lovgren, 1965; Newell et al., 1969). Cells were harvested from the filters at the desired time or morphological stage of development by vortexing the filters directly in buffer and then isolating the cells by centrifugation.

Development in Liquid Suspension. Alternatively, in some experiments development was initiated by resuspending the washed amoebae in SORPHOS at  $2 \times 10^7$   $\text{ml}^{-1}$  and shaking them at 22°C on a 170rpm orbital shaker until the desired time of development was reached.

In experiments where the calcium mobilization inhibitor, TMB-8, was used, cells were resuspended at  $10^8$   $\text{ml}^{-1}$  in SORPHOS with or without the inhibitor and were shaken at this density for the treatment time indicated. All manipulations involving cells used in calcium-related experiments were conducted in plastic vessels to preclude any complication by calcium which is believed to leach from glass containers.

Slug Development on Agar. Environmental conditions of low ionic strength and low buffering capacity are necessary to induce slug formation and sustained slug migration (Slifkin and Bonner, 1952; Newell et al., 1969; Devine et al., 1982). This environment was created by washing cells from clearing mass plates in five cycles of deionized water at 4°C and then plating a streak of thick cell suspension onto 2%(w/v) Noble agar (Difco) in deionized water. The streak was approximately 5cm in length along the boundary of a 90mm petri dish and a drain hole (0.5cm) was bored in the narrow agar strip between the streak and the petri dish wall. Development and sustained migration

cultures, 200ml samples of axenic media were inoculated to  $5 \times 10^4$  cells  $\text{ml}^{-1}$  and shaken at 170rpm in one litre flasks at  $22^\circ\text{C}$ .

#### B Maintenance of Stocks

Strains being used routinely were maintained on SM agar by cloning every four or five days by spreader dilution. This employed a series of three SM agar plates each inoculated with 0.2ml of a suspension of K. aerogenes. A drop of amoebal spore suspension was added to the first plate and spread with a sterile glass spreader over this plate and successively over the other two plates without sterilization of the spreader between plates. The carry-over of cells on the spreader produced isolated cells or spores (and subsequently clones) on the second or third plate (Wallace and Newell, 1982).

Primary stocks were maintained as spores, collected in horse serum, dried on silica gel, and stored at  $4^\circ\text{C}$  (Perkins, 1962). To initiate a new clonal culture, the silica gel stock was mixed with a small volume of ice-cold sterile SS and the supernatant diluted by the spreader dilution cloning technique.

In cases where the strain did not sporulate well, stocks were maintained frozen as vegetative amoebae in horse serum containing 5%(v/v) dimethylsulphoxide in plastic straws immersed in liquid nitrogen (Williams and Newell, 1976).

#### C Initiation and Development

All cells, except the axenic strain, were grown as "mass plates". Lawns of bacteria and slime mould inocula were plated onto SM nutrient agar at densities (usually  $10^5$  amoebae per plate) allowing uniform clearing of the bacterial lawn by the amoebae in 40-48 hours (72hr for D. lacteum). Throughout this study, a haemocytometer was used to determine cell density.

Development on Millipore Filters. Development was initiated by

## D Strains

The basic Dictyostelium discoideum strain used for most of these studies was NC4 (D.d.B) obtained from Dr. M. Sussman, derived from the original NC4 isolate of Raper (1935). Strain XP55 is a cycloheximide resistant (cycA5) derivative with an inability to grow on Bacillus subtilis (bsgA5) and is one of the basic parental stocks used in this laboratory for genetic studies (Ratner and Newell, 1978). The "brainy" mutant NP550 was derived from NC4 by N-methyl-N'-nitro-N-nitrosoguanidine (NTG) mutagenesis. The methanol resistant derivative of this mutant, NP551, was obtained by selective plating on 2% methanol SM agar. The axenic mutant, AX3, was also derived from NC-4 after NTG mutagenesis and selection in axenic media (Loomis, 1971). The "streamer" (stm) mutants NP368 (stmF406) and NP377 (stmF411) were derived from XP55 by NTG mutagenesis and carry mutations at the indicated loci (Ross and Newell, 1979,1981).

Dictyostelium lacteum was a gift from Dr. T.M. Konijn, Zoological Laboratory, University of Leiden, The Netherlands.

The bacterial food source for all experiments was Klebsiella aerogenes strain OXF1, a cobalt-resistant derivative of strain 1033 (Williams and Newell, 1976). Fresh bacterial stocks were prepared weekly from an isolated clone and the stock was renewed monthly from frozen stocks.

## 2.2 METHODS

### A Growth Conditions

All strains of D. discoideum were grown in the dark in association with K. aerogenes at 22°C on SM nutrient agar. Other species were cultured under the same conditions. The D. discoideum axenic mutant, AX3, was maintained on SM as described above but was mass cultured in axenic liquid medium defined in the Materials section. For the liquid

Lower Pad Solution (Sussman and Lovgren, 1965)

KCl	1.5g
MgCl <sub>2</sub> ·6H <sub>2</sub> O	0.5g
Dihydrostreptomycin sulphate	0.5g
KH <sub>2</sub> PO <sub>4</sub>	5.45g

Add 900ml deionized water, adjust pH to 6.5 with KOH and then adjust volume to one litre with further deionized water.

Upper Pad Solution (Newell et al., 1969)

Upper pad solution is 1M phosphate buffer made by combining 12.2ml 1M Na<sub>2</sub>HPO<sub>4</sub> and 87.8ml of 1M KH<sub>2</sub>PO<sub>4</sub> and adjusting pH to 6.0 with the appropriate buffer component.

Liquid Broth (Bacterial Cultures)

Yeast Extract	5g
Bacto-peptone	10g
NaCl	5g
1M Tris-HCl, pH 8.0	10ml
1M MgSO <sub>4</sub>	1ml
Deionized water	to 1 litre

Bacterial Freezing Solution (McFall et al., 1958)

KH <sub>2</sub> PO <sub>4</sub>	9g
K <sub>2</sub> HPO <sub>4</sub>	10g
Na Citrate	2.25g
1M MgSO <sub>4</sub> ·7H <sub>2</sub> O	1.83ml
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	4.5g
Glycerol	220g
Deionized water	to 500 ml

For use dilute ten fold by adding nine parts of liquid broth containing cultured bacterial cells. Aliquot bacterial suspension to small sterile vials and store at -70°C.

#### KP6 Phosphate Buffer

Potassium phosphate buffer pH6.0 (KP6) was made up as a 10X solution containing 87.7ml 500mM  $\text{KH}_2\text{PO}_4$  and 12.3ml 500mM  $\text{K}_2\text{HPO}_4$  and diluted when required.

#### KMMP Agar (Newell and Ross, 1982)

KCl	1.0g
MgCl	0.2g
MnCl <sub>2</sub>	0.2g
KP6 Buffer, 10X	40ml
Difco Bacto Agar	10g
Deionized Water	960ml

Gently boil until agar is completely dissolved then dispense 5ml aliquots into 50mm petri dishes. To prevent precipitation of salts it is essential to have the salts completely dissolved before adding the agar.

#### Buffered Agar (Chemotaxis agar)

This agar contained 2g Oxoid purified agar per 100ml of SORPHOS. Additions of attractants were at concentrations cited in the text.

#### Axenic Liquid Media (Watts and Ashworth, 1970)

Oxoid Bacteriological Peptone	14.3g
Oxoid Yeast Extract	7.15g
D-Glucose	15.4g
$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$	1.28g
$\text{KH}_2\text{PO}_4$	0.49g
Dihydrostreptomycin	250mg
Deionized Water	to 1 litre

Final pH6.7.

## 2 MATERIALS AND METHODS

### 2.1 MATERIALS

#### A Chemicals

All chemicals used in this study are tabulated in Appendix 1.

#### B Equipment

Appendix 2 contains a list of equipment used.

#### C Media

Standard Media (SM). The composition of this agar media was based on that of Sussman (1966).

Glucose	10g
Difco Bactopeptone	10g
MgSO <sub>2</sub> .7H <sub>2</sub> O	1g
KH <sub>2</sub> PO <sub>4</sub>	2.2g
K <sub>2</sub> HPO <sub>4</sub>	1g
Oxoid Yeast Extract	1g
Oxoid Agar (Technical No.3)	12g
Deionized Water	to 1 litre

Final pH 6.5. The medium was autoclaved at 15 lb in<sup>-2</sup> for 20min and dispensed in 33ml aliquots into petri dishes using a Petrimat automatic plate pourer.

#### SS Salts Solution (Bonner, 1947)

NaCl	0.6g
KCl	0.75g
CaCl <sub>2</sub> .H <sub>2</sub> O	0.3g
Deionized Water	to 1 litre

#### Sorenson's Phosphate Buffer 17mM, pH6.15 (SORPHOS)

KH <sub>2</sub> PO <sub>4</sub>	231.2mg
Na <sub>2</sub> HPO <sub>4</sub> .2H <sub>2</sub> O	19.6mg
Deionized Water	to 100 ml

30ml of 2% buffered agar and various chemoattractants as described in the text. Cells were harvested from the growth margin of clones, streaks, or from early clearing mass plates and washed four times in SORPHOS. The supernatant was discarded, and the final pellet vortexed into a thick slurry (approx  $10^9$  cells  $\text{ml}^{-1}$ ). In each petri dish 1 $\mu$ l cell drops were deposited in a set pattern on the agar surface. The degree of chemotactic movement was recorded by photography using dark field optics (Gross, et al., 1976; Ross and Newell, 1981).

The "directional" chemotaxis test monitored the directed movement of cells up a gradient of chemoattractant placed on only one side of cell droplets (Finney, et al., 1979; Varnum and Soll, 1981; Nandini-Kishore and Frazier, 1981). An agar substratum was attached to the surface of a microscope slide. Slides were first washed in chromic acid, rinsed in deionized water, and dried thoroughly with lint-free tissues. The slides were painted four times with 0.5% (w/v) molten agar solution in deionized water, allowing the slide to dry thoroughly between each painting; they were then coated with a 2-3mm surface of buffered agar by a casting process in 90mm petri dishes. For the casting process, petri dishes were partially filled with 20ml 2%(w/v) water agar (any type) and the agar was allowed to set. This provided a level surface on which to cast the slide coating. Without moving the petri dishes, the slides were laid onto the agar surface and 15ml of partially cooled 2%(w/v) buffered agar was poured over the slide and agar surfaces. After the second agar had solidified, the slides were cut from the agar and stored in a humidified chamber. To further prepare the slides for the chemotactic assay, two narrow troughs, 2mm x 50mm were cut in the agar with a standard immunoelectrophoresis template cutter. Variable concentrations of chemoattractants in 150 $\mu$ l aliquots were added to one slide trough and SORPHOS to the control trough, the slide was allowed to stand 15min in a sterile-flow hood and then 0.2-

0.3µl dense cell spots (approx.  $10^9$  ml<sup>-1</sup>) were evenly spaced between the troughs. Chemotactic migration was recorded by photography through dark field optics as for the "spreading" assay.

#### M Receptor Binding Assays and Competitive Binding Assays

Folate Binding to Whole Cells. A mixture of 10µl 6.5mM 8-azaguanine in deionized water, 30µl SORPHOS or 30µl 0.47mM folate, and 50µl cells (washed and resuspended at  $10^8$  ml<sup>-1</sup>) was added to the reaction tubes. The binding reaction was conducted at 4°C and was initiated by the addition of 40µl [<sup>3</sup>H]folate to obtain final reaction concentrations of 30-1500nM folate. By two minutes the binding reaction was at equilibrium and was terminated by layering 100µl reaction mixture onto 150µl AR20:AR200 silicone oil (1:1, Wackerchemie) in 400µl long form Eppendorf tubes and centrifugation at 8000g for 30sec (Roos et al., 1975). Cell pellets were recovered for scintillation counting by clipping the tube tips directly into counting vials and adding Unisolve I scintillant. Non-specific binding controls were those that contained the 0.47mM folate in place of the SORPHOS buffer. Binding results were analysed by Scatchard plots (Scatchard, 1949) and line fit was by least squares linear regression analysis.

Folate Binding to Cytoskeletons. For [<sup>3</sup>H]folate binding to cytoskeletons a modified method of Galvin et al. (1984) was followed. Crude cytoskeleton extracts were prepared by suspending cells at  $10^8$  ml<sup>-1</sup> in 20mM phosphate buffer (pH6.1) containing 0.6mM 8-aza guanine and then lysing the cells with 1/10 volume of 10% Triton X-100 and 10mM EGTA in 20mM phosphate buffer. Cell lysis was complete within 2min at room temperature and this crude cytoskeleton extract was used immediately in the binding assay. For the non-specific binding controls, the cytoskeleton preparative solution contained additionally 0.1mM folate.

A 200µl sample of cytoskeleton extract was added to 40µl [<sup>3</sup>H]folate at 30-1500nM (final reaction concentrations) and allowed to incubate on

ice for 2min. The reaction was terminated at 2min by layering duplicate 100ul samples onto 150ul oil (heavy mineral oil:silicone fluid 550; 2.6:1) in 400ul Eppendorf tubes and spinning at 8000g for 1min. The Triton insoluble cytoskeleton accumulated in the pellet and was analysed by scintillation counting as for the whole cells above.

Competitive Binding Assays. A competitive binding assay was used to test the ability of folate analogues to inhibit the binding of [<sup>3</sup>H]folate to the folate receptor. For this assay, whole cells were prepared as for the normal binding assay and resuspended in SORPHOS at  $10^8 \text{ ml}^{-1}$ . Cells were incubated with a constant concentration (300nM) of [<sup>3</sup>H]folate and variable concentrations of the competitor. Reaction tubes on ice were partially filled with 30ul competitor (0.47mM to 4.7nM); 30ul 2.3mM 8-aza-guanine; 50ul cells; 40ul 1.4μM [<sup>3</sup>H]folate (reaction concentration = 300nM). Control tubes contained 30ul unlabelled 0.47mM folate or 30ul SORPHOS in place of the competitor. The binding reaction was terminated after 2min by centrifugation through silicone oil and the cell samples prepared for scintillation counting as described for normal whole cell binding assays.

Folate B-Site Binding Assay. It is possible to detect a small population of high-affinity, slow-dissociating folate binding sites by a recently published method (De Wit and Van Haastert, 1985; De Wit, et al., 1985). Cells were harvested from early clearing mass plates in the normal manner and resuspended at  $3.4 \times 10^8 \text{ cells ml}^{-1}$ . The entire assay including cell preparation and sample centrifugation was conducted at 4°C. A 50ul cell sample was incubated for 120s in 120ul of incubation solution containing 0.33mM 8-aza-guanine, 1nM-0.1mM competitor and 10nM [<sup>3</sup>H]folate in SORPHOS. After 120s a 150ul cell suspension aliquot was added to 1050ul of 25μM folate layered over 180ul of silicone oil (11:4; AR20:AR200, WackerChemie) and a 20ul bottom cushion of 8% sucrose in a

1.5ml Eppendorf tube. The labelled ligand dissociated from fast dissociating receptors during a 60s dissociation period, then cells were separated from the dissociating solution (25 $\mu$ M folate) by centrifugation through the oil for 30s at 8000g. Sample tubes were placed on dry ice to freeze both the upper liquid layer and the sucrose layer. Cells were recovered for radioactivity analysis by cutting off the tips of the Eppendorf tube with a hot Nichrome wire cutter. Sample-containing tips were suspended in 5ml of Unisolve I, vortexed to resuspend and solubilize the pellet and radioactivity was measured on a LKB Rack Beta scintillation counter.

cAMP Binding to Whole Cells. This assay followed the well-established method of Green and Newell (1975) with cell recovery through silicone oil as described above. The general protocol is identical to that for folate binding with the obvious reagent alterations. Developing cells from shaker culture, washed and resuspended at  $7 \times 10^7$  ml<sup>-1</sup>, were added in 50 $\mu$ l aliquots to a reaction mixture containing 10 $\mu$ l 30mM DTT, 30 $\mu$ l SORPHOS or 30 $\mu$ l 3mM cAMP, 40 $\mu$ l [<sup>3</sup>H]cAMP (1.0-700nM reaction concentration). After equilibrium binding was reached (1min), 100  $\mu$ l aliquots were layered onto silicone oil, and samples prepared as described above.

#### N Folate Deaminase Assay

The folate deaminase activity in whole cells was determined by separating [<sup>3</sup>H]folate from deaminated [<sup>3</sup>H]deaminofolate ([<sup>3</sup>H]DAFA) using the cation exchanger SP-Sephadex (De Wit, 1982). Briefly, the basis of this assay is that folate at pH2.0 will have a positive charge on the NH<sub>3</sub><sup>+</sup> moiety at position 2 of the pteridine ring (see chapter 3) whereas the deaminated form will have an hydroxyl at this position and therefore have a neutral or negative charge. SP-Sephadex, a cation exchanger,

will bind cations; therefore, it will bind a large proportion of the [ $^3\text{H}$ ]folate in this batch method but will not bind [ $^3\text{H}$ ]DAFA. So an increase in supernatant radioactivity would reflect an increase in folate deaminase activity.

To replace the initial  $\text{Na}^+$  counter-ion with  $\text{NH}_4^+$ , 0.8g SP-Sephadex beads were pre-swollen overnight in 100ml 1M ammonium formate followed by washing on a scintered glass funnel with 200-300ml of 1M ammonium formate and then one litre of 10mM ammonium formate buffer, pH2.0. The 10mM ammonium formate buffer was prepared by adding concentrated formic acid to 10mM ammonium formate to yield pH2.0. The beads were resuspended in approximately 10ml of formate buffer to yield a slurry where the settled resin was 50% of the total volume. The beads were distributed in 150 $\mu\text{l}$  aliquots to 1.5ml Eppendorf tubes and then spun for 30sec at 8000g; 70 $\mu\text{l}$  of the buffer supernatant was withdrawn from each tube, leaving only the bead pellet and a small volume of buffer. The beads were cooled on ice, ready for use in the assay.

Vegetative or developing cells were washed and suspended at  $10^8$  cells  $\text{ml}^{-1}$  in 20mM phosphate buffer ( $\text{Na}_2/\text{K}$ ), pH6.0. The enzymic reaction was initiated by mixing in 1.5ml Eppendorfs, 50 $\mu\text{l}$  cells, 30 $\mu\text{l}$  350nM [ $^3\text{H}$ ]folate, and 30 $\mu\text{l}$  of the deaminase inhibitor, 1.5mM 8-azaguanine, or 30 $\mu\text{l}$  deionized water. After incubation for 2min on ice, cells were spun down at 8000g for 30sec and 100 $\mu\text{l}$  of supernatant digest was layered onto 80 $\mu\text{l}$  of settled SP-Sephadex beads on ice. The supernatant was absorbed with the beads for 2min and then the beads were pelleted by an 8000g 30sec spin. Duplicate 20 $\mu\text{l}$  supernatant samples were transferred to scintillation vial inserts, pre-filled with 4ml of Unisolve I scintillant, and radioactivity measured on a LKB Rack Beta scintillation counter. Controls included: a) no enzyme (i.e., 50 $\mu\text{l}$  buffer in place of cells), b) no [ $^3\text{H}$ ]folate bound (80 $\mu\text{l}$  ammonium formate buffer in place of SP-Sephadex beads) c) no deaminase inhibitor (30 $\mu\text{l}$

deionized water in place of 8-aza-guanine).

O EDTA Resistant Cell Cohesion Assay

Developing cells were analysed for ability to develop EDTA resistant cell adhesion sites, contact sites A, by the method of Gerisch, (1961) as modified by Glazer and Newell (1981). Cells were harvested in 1.5ml samples from shaker culture at the desired stage of development and vortexed vigorously for 15sec to dissociate possible existing aggregates. Separate 0.5ml aliquots from the 1.5ml sample were diluted with either 0.5ml SORPHOS or 0.5ml 20mM EDTA in SORPHOS. Mixtures were vortexed and allowed to stand for 2min. Samples from both mixtures were applied to opposite chambers of a haemocytometer and cell aggregates were tabulated by size (i.e. cell number, singles, 2-5, 6-10, 11-15, 16-50, >50).

P cGMP Radioimmunoassay

The ability of certain chemoattractants to induce transient increases in cGMP in developing slime mould cells was evaluated by a published method (VanHaastert, et al., 1981) using a radio-immunoassay kit from Amersham.

Vegetative cells, or cells harvested at desired stages of development, were suspended at  $10^8 \text{ ml}^{-1}$  and aerated with 95%  $\text{O}_2$ , 5%  $\text{CO}_2$  for 5min while on ice. A 3ml cell sample was then transferred to a  $170 \text{ rev min}^{-1}$  rotary shaker at  $22^\circ\text{C}$  and pulsed four times at 5min intervals with 100 $\mu\text{l}$  of 1.55mM folate to synchronize the cells. Five minutes after the fourth pulse, 100 $\mu\text{l}$  cell aliquots were placed in 1.5ml Eppendorf tubes on a Vibrax shaker and agitated at 1400rpm. Duplicate 100 $\mu\text{l}$  samples were stimulated for 0-31sec with 20 $\mu\text{l}$  0.3mM folate and the response interrupted at selected time points with 100 $\mu\text{l}$  ice cold 3.5% (v/v) perchloric acid. The 0sec control involved cell lysis with perchloric acid prior to folate stimulation. After cell lysis, sample

tubes were immediately transferred to ice, neutralized with 50 $\mu$ l 50% saturated  $\text{KHCO}_3$  and then returned to the Vibrax and shaken for 5min to release  $\text{CO}_2$ . Tubes were then capped and the  $\text{KClO}_4$  removed from the supernatant by centrifugation at 8000g for 2min. Fifty microlitre aliquots of supernatant were transferred to fresh 1.5ml Eppendorf tubes and the cGMP content determined by first adding 25 $\mu$ l of [ $^3\text{H}$ ]cGMP and 25 $\mu$ l of anti-cGMP antiserum prepared as by VanHaastert, et al. (1981). Samples were vortexed and stored on ice at 4°C for at least 1.5hr. The antibody-cGMP complex was precipitated by adding 500 $\mu$ l ice-cold, 60% saturated ammonium sulphate, and centrifugation at 8000g for 2min. The supernatant was carefully removed by vacuum aspiration and any remaining liquid absorbed with cotton-wool buds. The pellet was dissolved in 75 $\mu$ l deionized water and the sample plus Eppendorf tube with cap removed was dropped directly into a scintillation vial. Fifteen millilitres of toluene/Triton X-100/PPO scintillant was added to each vial, vials were fully mixed and radioactivity determined on an LKB Rack Beta scintillation counter.

#### Q Paper Chromatography of Folates

Folate and folate derivatives were identified and purified by paper chromatography (Wurster and Butz, 1980). Folates, dissolved at 1mM in 0.5%  $\text{Na}_2\text{CO}_3$ , were spotted in 50 $\mu$ l samples to Whatman 3MM (0.38mm thick) paper strips (12.5cm X 50.0cm) at 2.5cm intervals along one end of the strip. Chromatograms were run for 3-3.5hr in a dark, humidified chamber with 0.5%  $\text{Na}_2\text{CO}_3$  as solvent, dried, and the folate spots detected by long wavelength UV illumination (Gadsen et al., 1960).

For preparative paper chromatography, 300 $\mu$ l samples of folates (1mM in 0.5%  $\text{Na}_2\text{CO}_3$ ) were applied as 7.5cm streaks on one end of the paper strip (dimensions as above). Chromatograms were run as above, allowed to dry, bands detected by UV illumination, and the folates recovered by

centrifugal elution from the paper with 0.5%  $\text{Na}_2\text{CO}_3$ , buffer, or water. To elute the samples, the separated bands seen under UV light were cut from the paper, rolled in aluminium foil strips (slightly narrower and longer than the paper) (Edwards, 1979). The tightly rolled paper bullet was suspended in the mouth of a centrifuge tube, secured in a vertical position with tape, saturated with eluant and then centrifuged at 500g for 5min. The elution procedure was repeated four times and then the concentration of the eluted sample was measured by UV spectroscopy. A molar extinction coefficient of  $5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$ , the normal value for folates, (Blakley, 1969; DeWit and Konijn, 1983b) was employed.

#### R DIF Induced Stalk Cell Differentiation

Centre clearing mass plates were harvested and the cells washed four times in SS and suspended in stalk salts (10mM MES, 2mM NaCl, 10mM KCl, 1mM  $\text{CaCl}_2$ , 10mM cAMP, 200ug  $\text{ml}^{-1}$  streptomycin sulphate, 15ug  $\text{ml}^{-1}$  tetracycline, adjusted to pH6.2 with KOH) (Kay, 1982; Kopachik, et al., 1983). Two millilitre aliquots of the cell suspension were added to 5cm tissue culture petri plates. To determine the effect of DIF on in vitro stalk cell development, 150U of purified DIF (generous gift from Dr. R. Kay) was added per ml of stalk salts. After incubation for two days in a dark humidified chamber, amoebae and stalk cells were scored by phase-contrast microscopy.

#### S Cell Respiration Measured by Oxygen Electrode

The electrode apparatus consisted of a 10ml water-jacketed reservoir equipped with a Clark-type oxygen electrode (as described by Wood, 1978). The recorder and electrode were synchronized and the maximum and zero levels set by saturating SORPHOS with compressed air and oxygen-free nitrogen, respectively.

Amoebae were harvested from clearing mass plates, washed four times in SS and resuspended at  $5 \times 10^7$  cells  $\text{ml}^{-1}$  in SORPHOS. They were then

placed on a rotary shaker for 40min at 22°C in the presence or absence of putative respiratory inhibitors. A ten millilitre sample of amoebal suspension was placed in the reservoir at 25°C, buffer and cells saturated with oxygen by bubbling with compressed air and then the respiration rate recorded. Full oxygen saturation resulted in a solution of 240µmole oxygen ml<sup>-1</sup> and therefore it was possible to calculate oxygen consumption from the recorder trace (Chappell, 1964).

#### T High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography was conducted on a Partisil 10/25 SAX Whatman column using an Altrex pump model 100A and an Altrex 165 variable wavelength detector as previously described (Kakebeeke, et al., 1980a). The solvent, 50mM phosphate buffer (Na/Na<sub>2</sub>) pH 7.0, was passed through the column at a flow rate of 1ml min<sup>-1</sup>. Folate derivatives were applied to the column as 10µl samples (10µg ml<sup>-1</sup>). Samples separated by HPLC were detected by UV absorption at 254 and 280nm.

#### U Statistical Data Analysis

Distribution free plot (non-parametric) analysis of binding data employed the micro-computer program of Crabbe (1985) modified in our laboratory to directly accommodate Scatchard binding data. A copy of this BASIC program has been included in Appendix 3.

Line fitting of all Scatchard plots was by least squares linear regression analysis.

## *Chapter 3*

### 3 FOLATE-SPECIFIC RECEPTORS ON THE CELL SURFACE OF VEGETATIVE DICTYOSTELIUM DISCOIDEUM AMOEBAE

#### 3.1 Introduction

Specific receptors for chemotactic ligands are essential for cellular communication. In order to demonstrate folate-initiated transduction at the 12hr tipped-aggregate stage of development, it seemed important to determine if there were folate receptors present or reappearing at this stage of development. The published literature on folate receptor assays, however, was contradictory so initial experiments were concerned with the development of a valid and reliable assay method.

Several workers have reported the presence of folate receptors in Dictyostelium discoideum through a variety of binding assays. Figure 3.1 schematically reviews these early studies. Wurster and Butz (1980) (figure 3.1a) first demonstrated folate-specific receptors using a silicone oil centrifugation method for ligand/cell separation and reported 60,000 sites per vegetative cell with a dissociation constant ( $K_d$ ) of 150nM and a slightly curvilinear Scatchard plot. Their studies were somewhat complicated, however, by not inhibiting the membrane-associated folate deaminase (Pan and Wurster, 1978; Kakebeeke et al., 1980b). They determined that only 13% of the folate was deaminated by 30s when the binding was conducted at 0°C and therefore used a 30s binding period and low temperature to minimize the deaminase problem. Wurster and Butz (1980) also reported that the deaminated folate (2,4-hydroxy-2-deamino folate (DAFA)) and folate bind with nearly equal affinity to this receptor. Van Driel (1981) (figure 3.1a) employed a Millipore filter assay method at 12°C which was complicated by high non-specific binding backgrounds as well as the folate deaminase problem. With this assay he found 100,000-200,000 sites per cell with a  $K_d$  of

Figure 3.1 Schematic Review of Folate Binding Studies

- (a) Folate binding in the absence of a folate deaminase inhibitor.
- (b) Methotrexate as the binding ligand.
- (c) Polyspecific DAFA/folate sites and specific folate sites detected while using the folate deaminase inhibitor 8-aza-guanine.
- (e) Folate receptors that bind folate and DAFA with equal affinity detected while using 8-aza-guanine.

# Review of folate binding studies

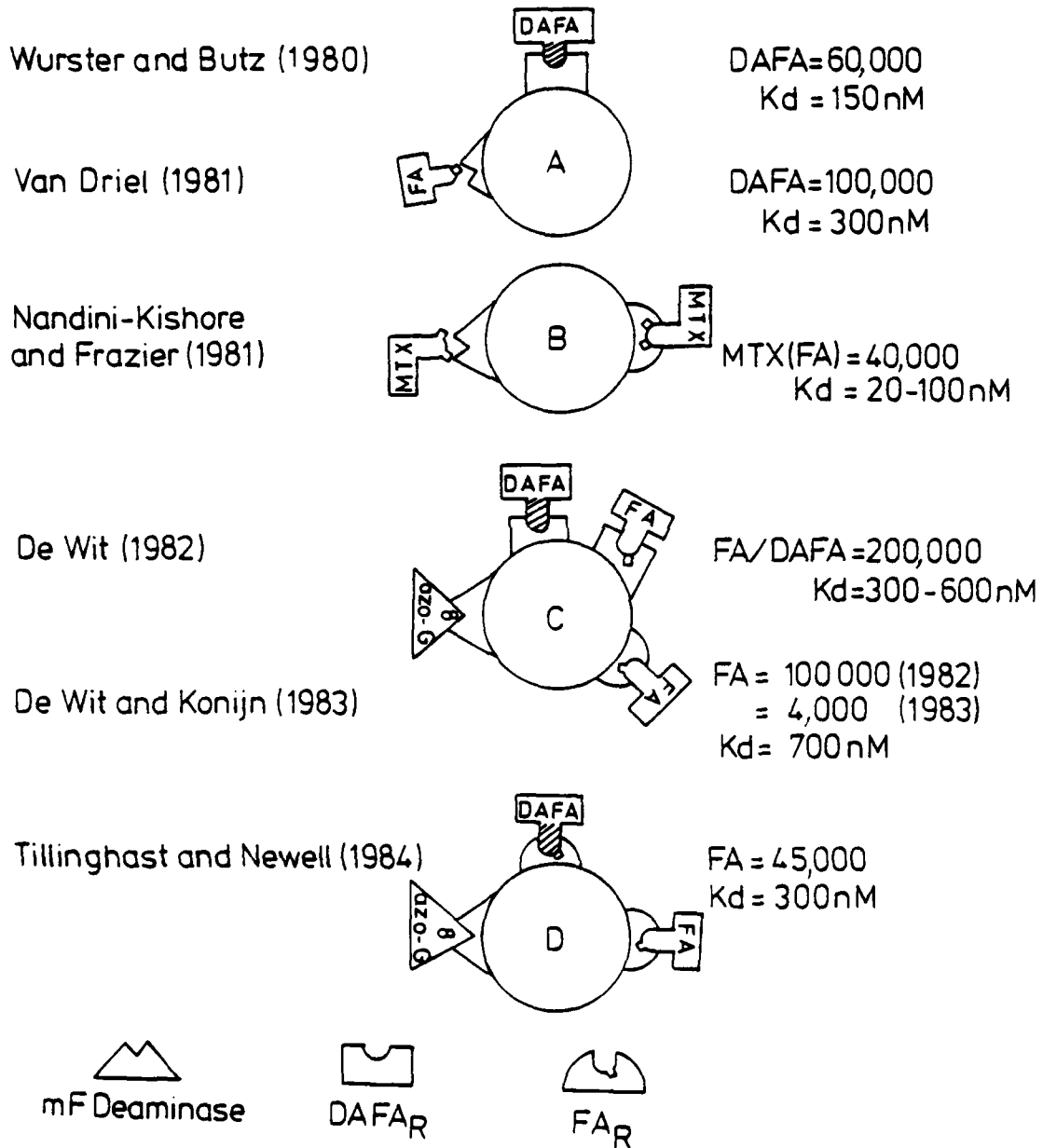


Fig 3.1

300nM and a highly curvilinear Scatchard plot. As suggested in figure 3.1a, it was impossible for these workers to know whether they were binding folate or whether they were actually binding the deaminated form of folate (DAFA).

Nandini-Kishore and Frazier (1981) attempted to overcome the deamination problem by studying binding of the folate analogue methotrexate which binds to Dictyostelium discoideum with an affinity similar to folate (Wurster and Butz, 1980) yet is not degraded by the folate deaminase (Nandini-Kishore and Frazier, 1981). Methotrexate proved to be a suitable alternative ligand and these workers detected 40,000 sites per cell with a variable  $K_d$  of 20-100nM and a linear Scatchard suggesting no degree of cooperativity or multiple affinity sites.

A third binding approach was used by De Wit (1982) when he inhibited the folate deaminase with 8-aza-guanine (Rembold and Simmersbach, 1969; Tsusue, 1971; Takikawa et al., 1979). From this report the folate binding seemed more complex. There appeared to be 200,000 sites per cell that bound either folate or DAFA with equal affinity ( $K_d=300-600nM$ ) and apparent negative cooperativity and an additional population of sites (100,000 per cell,  $K_d=700nM$ ) that preferentially bound folate in the presence of excess DAFA and showed a linear Scatchard. De Wit therefore suggested that the group of sites insensitive to DAFA competition, were the true chemotactic sites. To complicate the folate binding picture further, De Wit and Konijn (1983a) have since published a review showing only 4000 folate-specific sites per vegetative cell.

Using this historical background, I developed the folate binding assay used in this study. (Tillinghast and Newell, 1984). I chose to employ the silicone oil method (Roos, et al., 1975; Klein and Juliani, 1977) since it seemed technically less cumbersome, produced lower non-

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CELL COMMUNICATION DURING AGGREGATION AND DEVELOPMENT  
OF THE CELLULAR SLI. (U) AIR FORCE INST OF TECH  
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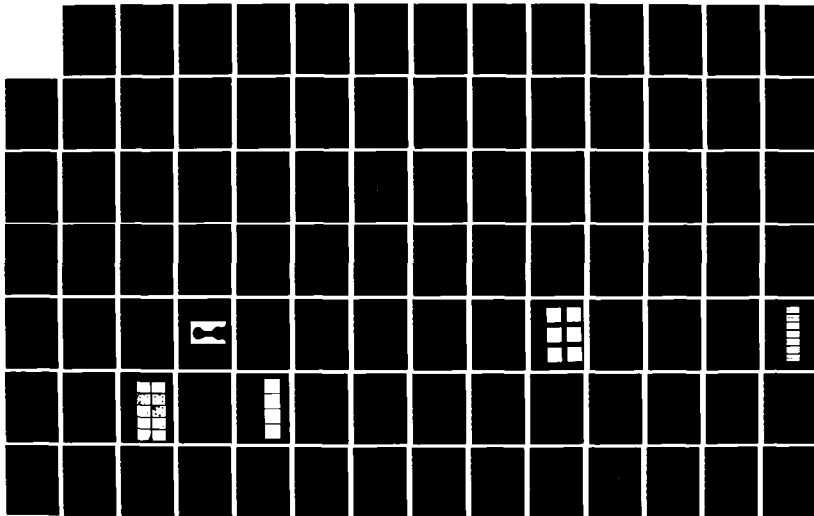
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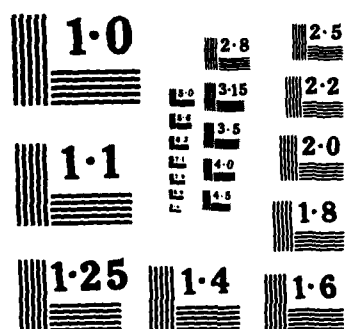
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specific backgrounds (Nandini, Kishone and Frazier, 1981), and has been cited as the method of choice for receptor binding studies involving ligand-receptor dissociation constants between 100 and 1000nM (Enna, 1984; Birdsall and Hulme, 1984).

This chapter presents the results from control experiments designed to optimize the folate binding assay. First, I found it necessary to synthesize DAFA and to evaluate the use of DAFA in detecting folate-specific receptors. I then assessed the use of 8-aza-guanine as a folate deaminase inhibitor and finally characterized the binding of folate to the cell-surface receptors of vegetative Dictyostelium discoideum amoebae.

### 3.2 Results

#### A Association of [<sup>3</sup>H]Folate With Cell-Surface Receptors

The kinetics of folate ligand association with vegetative amoebae is shown in figure 3.2. Within 30sec the association of 100nM [<sup>3</sup>H] folate with surface receptors reached an equilibrium plateau which remained constant for 5min. From this result, I concluded that the degree of binding of labelled ligand to cells could be measured at any time after 30s and that it would be equally valid to terminate the incubation period at 2min by spinning the cells through oil and determine equilibrium binding at this time.

#### B Competition Binding of [<sup>3</sup>H]Folate and Unlabelled Folate

A competitive binding assay was conducted to determine the concentration of excess unlabelled folate necessary to compete for all specific folate binding sites and therefore the concentration needed to determine non-specific binding levels. Washed amoebae from the vegetative phase were incubated with 100nM [<sup>3</sup>H]folate in the presence of variable concentrations of unlabelled folate from  $10^{-9}$ - $10^{-3}$ M (figure 3.3). The results showed that  $10^{-4}$ M unlabelled folate was sufficient to

Figure 3.2     Association of [<sup>3</sup>H]folate with Vegetative Amoebae

Cells were incubated with 100nM [<sup>3</sup>H]folate for various time periods (0-5min) and then separated from the supernatant by centrifugation through silicone oil. All data points were corrected for non-specific binding determined in the presence of 0.1mM folate. This data was obtained from a single experiment.

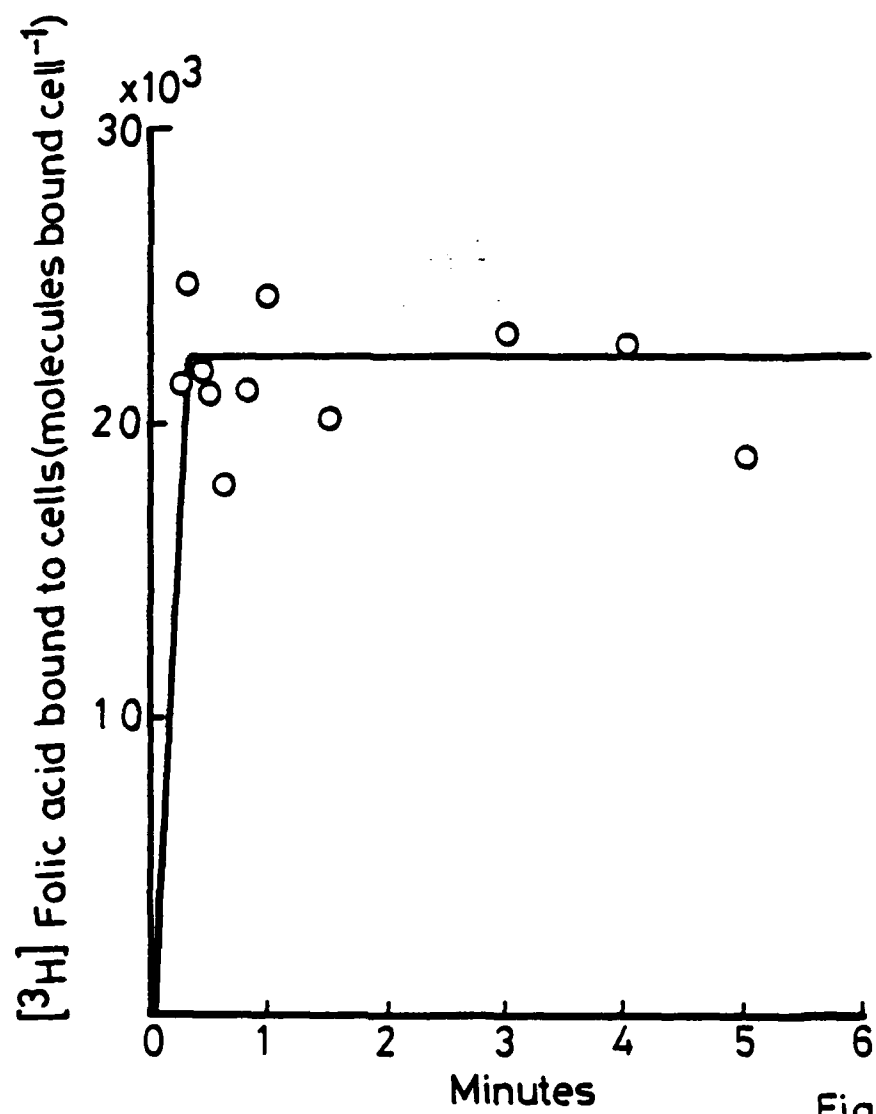


Fig 3.2

Figure 3.3    Competition Binding of [<sup>3</sup>H]folate and  
Unlabelled Folate

Cells were incubated in the presence of 100nM [<sup>3</sup>H]folate and various concentrations of unlabelled folate. This is a representative example of three separate experiments.

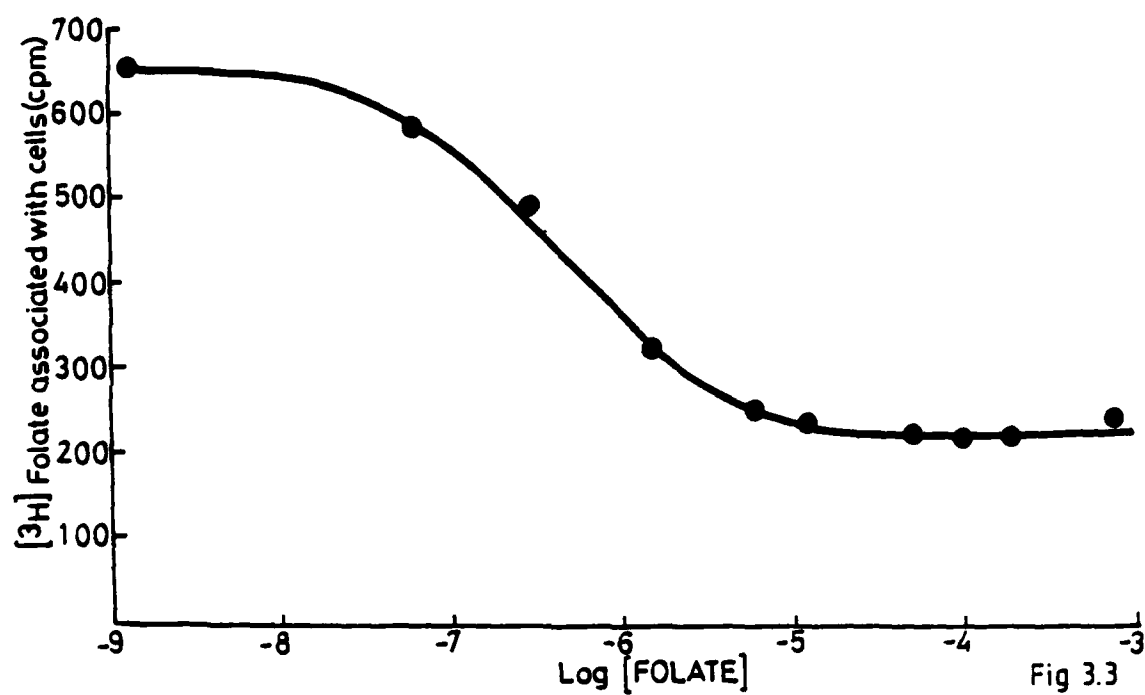


Fig 3.3

compete for all specific folate binding sites. I therefore used 0.1mM unlabelled folate to determine the non-specific binding levels in all subsequent experiments.

#### C Folate Deaminase Inhibition by 8-aza-guanine

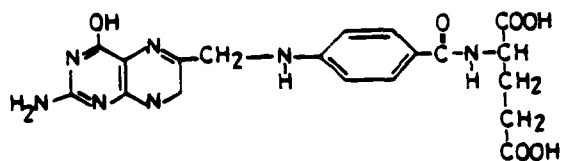
Several derivatives of guanine have been shown to inhibit pterin deaminase in rat liver and honeybee larvae (Rembold and Simmersbach, 1969), the sepiapterin deaminase in silkworms (Tsusue, 1971), the pterin deaminase in Bacillus megaterium (Takikawa, et al., 1979) and more recently the modified purine, 8-aza-guanine was shown to effectively inhibit the folate deaminase of Dictyostelium discoideum (De Wit, 1982). To determine the optimum concentration of 8-aza-guanine for inhibiting the Dictyostelium discoideum folate deaminase, I employed an assay described in detail in Chapter 2.

Washed amoebae from the vegetative phase possessing the membrane associated folate deaminase (Pan and Wurster, 1978; Kakebeeke et al., 1980b) were incubated with 100nM [<sup>3</sup>H]folate in the presence of various concentrations of 8-aza-guanine (figure 3.4a) and then separated from the supernatant digest by centrifugation. The supernatant was extracted with SP-Sephadex as described in Chapter 2 and then assayed for the presence of radioactive deaminated folate, [<sup>3</sup>H]DAFA, since any non-degraded [<sup>3</sup>H]folate was absorbed by the SP-Sephadex resin (figure 3.4b). It was found that concentrations of 8-aza-guanine above 0.1mM were sufficient to inhibit the folate deaminase when the assay was conducted at 0°C (on ice). From the results of this experiment, I chose to conduct the binding assays in the presence of 0.5mM 8-aza-guanine at 0°C.

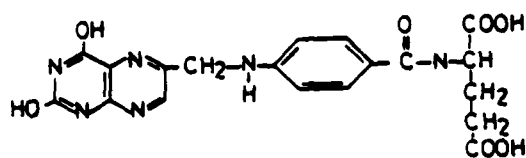
Figure 3.4     Folate Deaminase Inhibition by 8-aza-guanine

- a) Chemical structures of the folate, the deaminated analogue of folate, DAFA, and the deaminase inhibitor, 8-aza-guanine.
- b) Cells were incubated with 100nM [<sup>3</sup>H]folate in the presence of various concentrations of 8-aza-guanine and then [<sup>3</sup>H]DAFA was measured in the supernatant after SP-Sephadex extraction. This is a representative example of three experiments.

Folic acid



2-Deamino-2-hydroxy-folic acid



8-Aza - Guanine

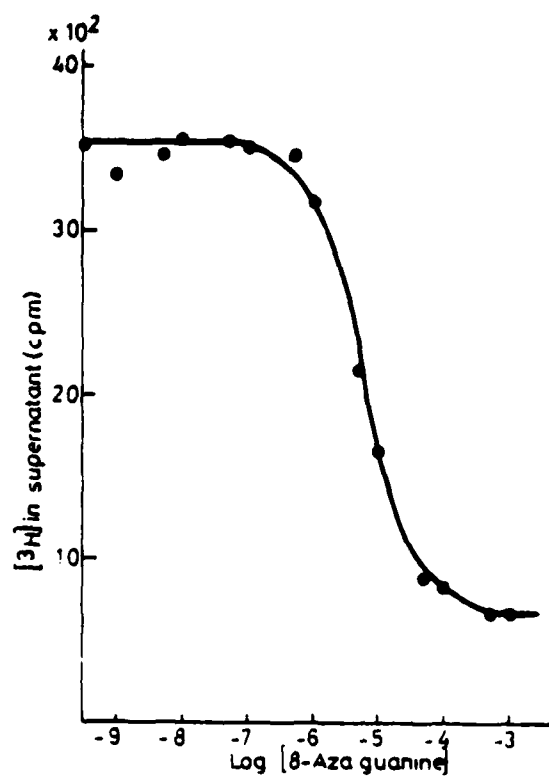
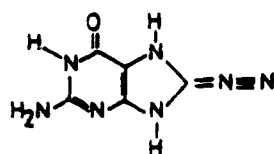


Fig.34

D Synthesis, Identification, and Purification of  
2,4-hydroxy-2-deamino-folate (DAFA)

Since De Wit (1982) reported that specific folate receptors could only be detected in the presence of 50 $\mu$ M DAFA, it seemed essential to obtain a source of this product. DAFA is not commercially available so other workers obtained gifts from chemical companies (Wurster and Butz, 1980) or prepared DAFA enzymatically (De Wit, 1982) by using the D. lacteum extracellular deaminase known to produce only the deaminase and not the N<sup>10</sup>-splitting enzyme (Kakebeeke et al., 1980a). A third alternative was to synthesize DAFA by the method of Angier et al. (1952) (used also by Kakebeeke, et al., 1980a); I chose this latter option since it provided the advantage of obtaining high product yields of a relatively pure compound.

The DAFA which I synthesized (figure 3.5) was identified by UV spectroscopy and comparison of the resultant spectra of the intermediate compound N<sup>10</sup>-nitroso-2-deamino-2-hydroxyfolate and the putative DAFA product (figure 3.6) with published reference spectra (Angier et al., 1952).

The synthesized DAFA product was compared to the DAFA produced by folate deamination with D. lacteum extracellular folate deaminase (figure 3.7). To obtain the extracellular enzyme extract, vegetative D. lacteum cells were suspended at 2x10<sup>7</sup> cells ml<sup>-1</sup> and shaken on a rotary shaker (170rpm) for 3hr. Cells were removed from the supernatant by centrifugation followed by Millipore filter sterilization. A stock folate solution (0.1mM) was diluted to 50 $\mu$ M with an equal volume of unconcentrated D. lacteum extracellular enzyme extract. UV spectra obtained periodically during 65min (figure 3.7a) showed that digestion to DAFA was complete after 45-60min. The starting folate and enzymatically produced DAFA spectra are compared directly in figure 3.7b

Figure 3.5     DAFA Synthesis Scheme  
(Angier et al., 1952)

# 2,4-HYDROXY-2-DEAMINO-FOLATE (DAFA) SYNTHESIS

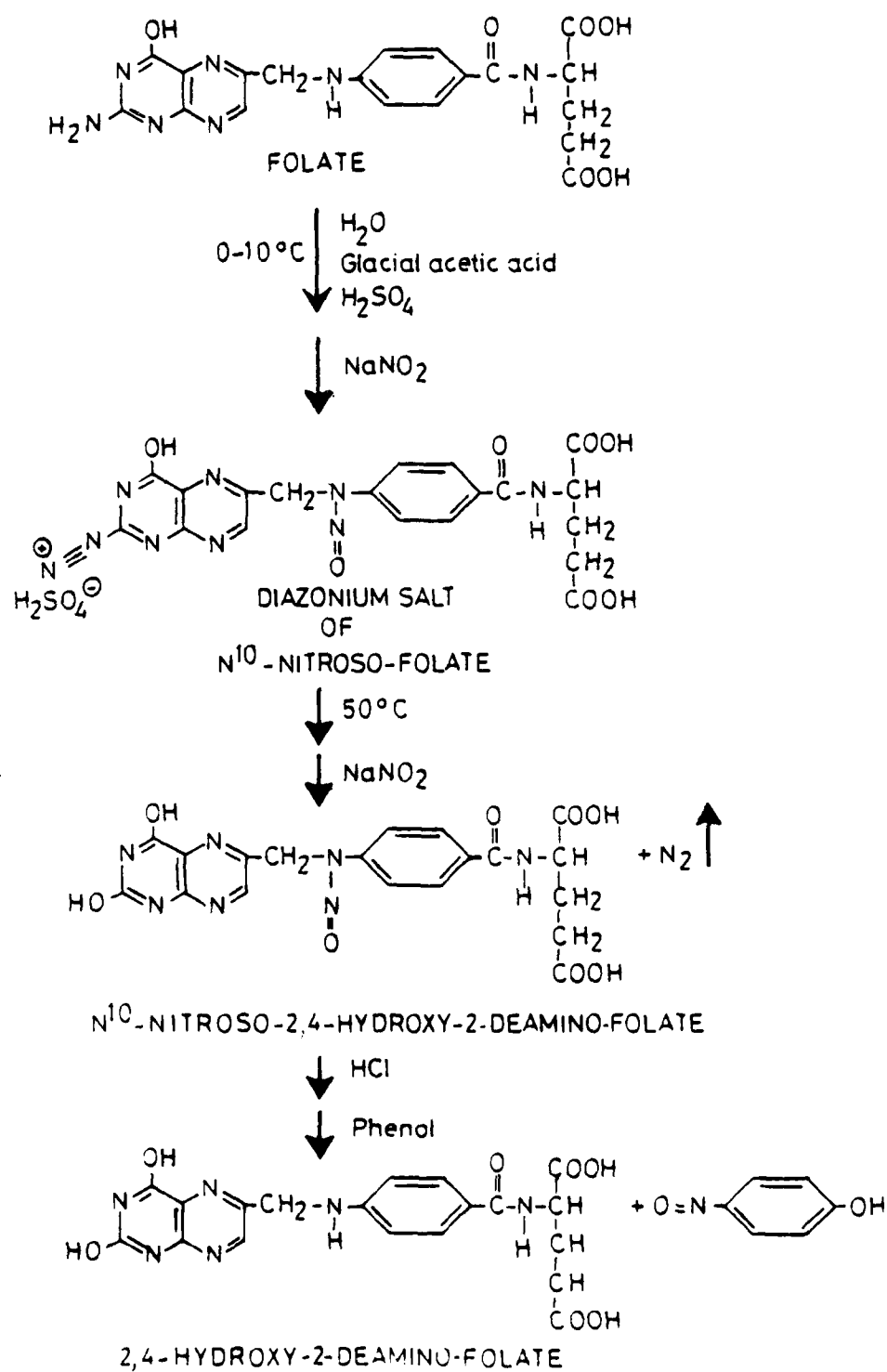


FIG 3.5

re 3.6    UV Spectra of Folate Derivatives in  
Various Solvents

1N HCl    (-----)

1N NaOH   (———)

1N NH<sub>4</sub>OH   (- - - - -)

The reduced absorption values in brackets  
apply only to the HCl solvent.

- a) Folate spectra.
- b) N<sup>10</sup>-nitroso-DAFA spectra.
- c) DAFA spectra.

#### 4 FOLATE RECEPTORS ON THE CELL-SURFACE AND CYTOSKELETON ARE RETAINED DURING DEVELOPMENT

##### 4.1 Introduction

The folate-elicited actin-accumulation response at 12hr of development (McRobbie and Newell, 1983, 1984a; chapter 1), suggests a developmentally regulated role for folate in later development. This developmental role could be controlled by regulation of the presence or absence of folate receptors. Therefore, I investigated the fate of folate receptors during development. Several preliminary and incomplete reports have previously approached this question (Wurster and Butz, 1980; VanDriel, 1981; Nandini-Kishore and Frazier, 1981; De Wit and Konijn, 1983a); the past results show a spectrum of responses from no loss of binding through 10hr of development to complete loss of receptors by 8hr.

An alternative means of folate receptor regulation would be developmental control of receptor-coupling to the motile-apparatus. Workers have suggested such a direct receptor-cytoskeleton link in human granulocytes (Jesaitis et al., 1984). Since the renewed folate response was detected as a cytoskeleton-related response, it seemed appropriate to determine the presence and fate of cytoskeleton-associated folate receptors.

##### 4.2 Results

###### A Folate Receptor Regulation during Development

The fate of folate receptors through 14hr of development is shown in figure 4.1. The number of receptors detected by the folate binding assay described in chapter 3 declined by 50% within 4hr after initiating starvation on filters and continued to decline, though somewhat more slowly, through 14hr of development. One possible explanation for the initial rapid decline in receptor number was that the [<sup>3</sup>H]folate was

## *Chapter 4*

that of folate. Thus, it was impossible to distinguish any specific folate receptors from the polyspecific DAFA/folate sites. Two possible explanations of this finding are that the synthesized DAFA was contaminated with folate or that the folate deaminase was not fully inhibited. Neither possibility seems likely. As reported, the DAFA was fully characterized and purified and HPLC showed no folate contamination (figure 3.8b). Control experiments (figure 3.4b) showed complete inhibition of the folate deaminase by 8-aza-guanine when used at 0.5mM and 0°C.

While the detectable folate receptors bind DAFA and folate with equal affinity, it remains unclear why DAFA is not a chemoattractant for vegetative amoeba (Van Haastert et al., 1982c; and Chapter 6). The original results of De Wit (1982) seemed to solve this problem, as chemotaxis could have been due simply to the folate specific receptors. However, since work reported here shows that the receptor is polyspecific, we must look for other explanations of this apparent anomaly.

In the next chapter I have employed the binding assay to answer several questions: 1) What is the fate of these folate receptors through development? 2) Do the receptors disappear with the chemotactic response and then reappear at the 12hr point in development? 3) As suggested for the cAMP receptor (Galvin et al., 1984), are there folate receptors on isolated cytoskeletons?

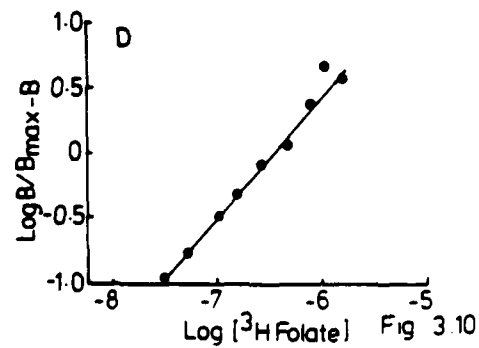
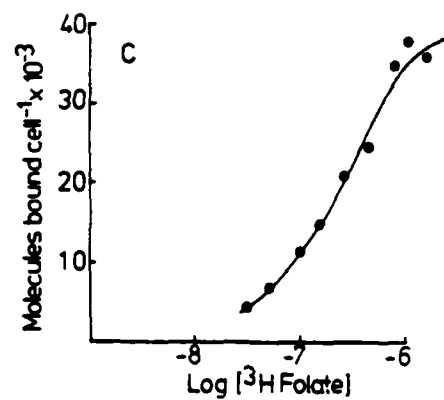
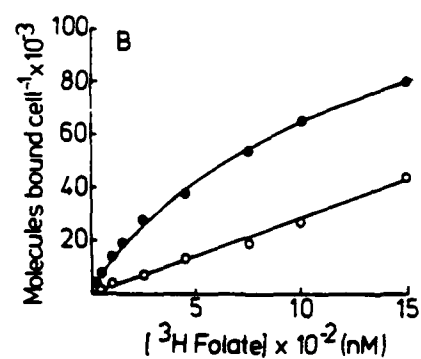
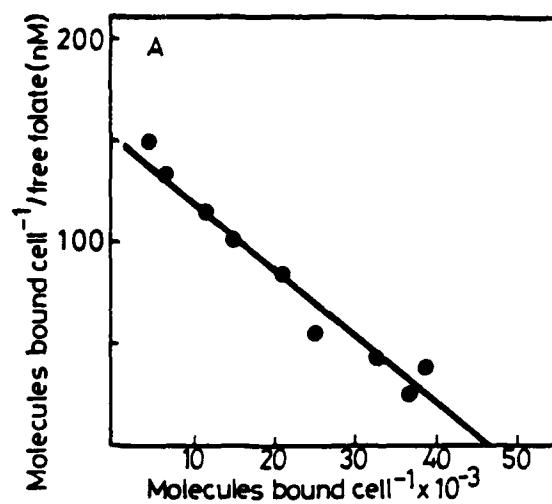


Figure 3.10 Folate Binding to Cell-surface Receptors  
of Vegetative Amoebae

(a) Scatchard analysis of folate binding

Cells were incubated with [ $^3\text{H}$ ]folate (30-1500nM) in the presence of 8-azaguanine (0.5mM). Non-specific binding controls contained additionally 0.1mM unlabelled folate. Line fit was by least squares linear regression analysis ( $r = 0.98$ , mean of three separate experiments).

(b) Saturation plot of folate binding

Total molecules bound cell $^{-1}$  (●) and the number of molecules bound non-specifically (○) are plotted against [ $^3\text{H}$ ]folate concentrations (30-1500nM).

(c) Klotz plot of folate binding

(Klotz, 1982)

Molecules bound cell $^{-1}$  versus free, unbound folate.

(d) Hill plot of folate binding

$B_{\text{max}}$  = total sites per cell  
(45,600, Scatchard plot)

$B$  = molecules bound cell $^{-1}$  at  
various concentrations.

both ligands have an equal affinity ( $K_d=300\text{nM}$ ) for the folate receptor detected by this assay. With this result, I deleted DAFA from all subsequent assays and conducted the assay as described in Chapter 2.

#### F Scatchard Plot Analysis of Folate Binding to the Cell-Surface Folate Receptor

The folate binding assay, as validated above, was employed to characterize folate binding to surface receptors on vegetative amoebae. Scatchard plot analysis (Scatchard, 1949) of [ $^3\text{H}$ ]folate (30-1500nM) binding in the presence of 8-aza-guanine (0.5mM) and at  $0^\circ\text{C}$  revealed 45-50,000 binding sites per cell with a dissociation constant of 300nM (figure 3.10a). The linear nature of the Scatchard plot suggests a single class of sites with a fixed affinity and no cooperativity (Scatchard, 1949; Rosenthal, 1967; Blondeau and Robel, 1975). Further analysis of the data showed that the sites were saturated with 1500nM [ $^3\text{H}$ ]folate (figure 3.10b). The Klotz plot (Klotz, 1982) has been suggested as an alternative analysis to confirm that the receptors are totally saturated, thereby ensuring that the Scatchard plot is a true estimate of total receptor number. This plot (figure 3.10c) appears to confirm that the receptor is fully saturated. The Hill plot slope is 1.0 (figure 3.10d) which also suggests a single class of fixed affinity receptors (Boeynaems and Dumont, 1975) and corroborates the Scatchard plot result.

#### G Discussion

With a revised folate binding assay that uses 8-aza-guanine (0.5mM) to block the membrane associated folate deaminase and excess folate (0.1mM) to measure non-specific binding values I detected 45,000 receptors on the surface of vegetative amoebae. I showed, contrary to the work of De Wit (1982), that DAFA bound with an affinity equal to

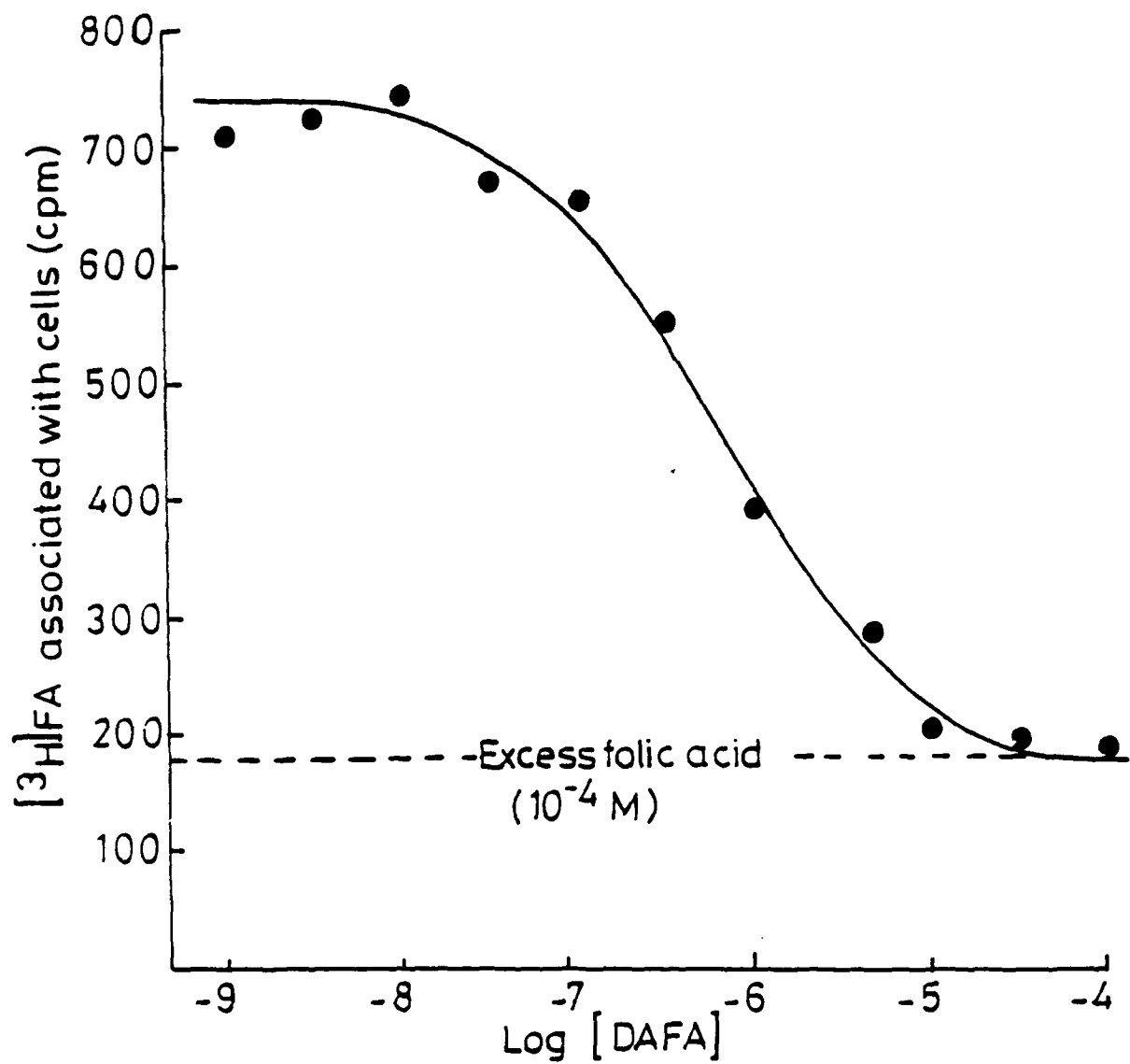


Fig 3.9

Figure 3.9    Competition Binding of [<sup>3</sup>H]folate and  
Unlabelled DAFA

Cells were incubated in the presence of 100nM [<sup>3</sup>H]folate and with various concentrations of synthesized and purified DAFA. This is a representative example of numerous experiments

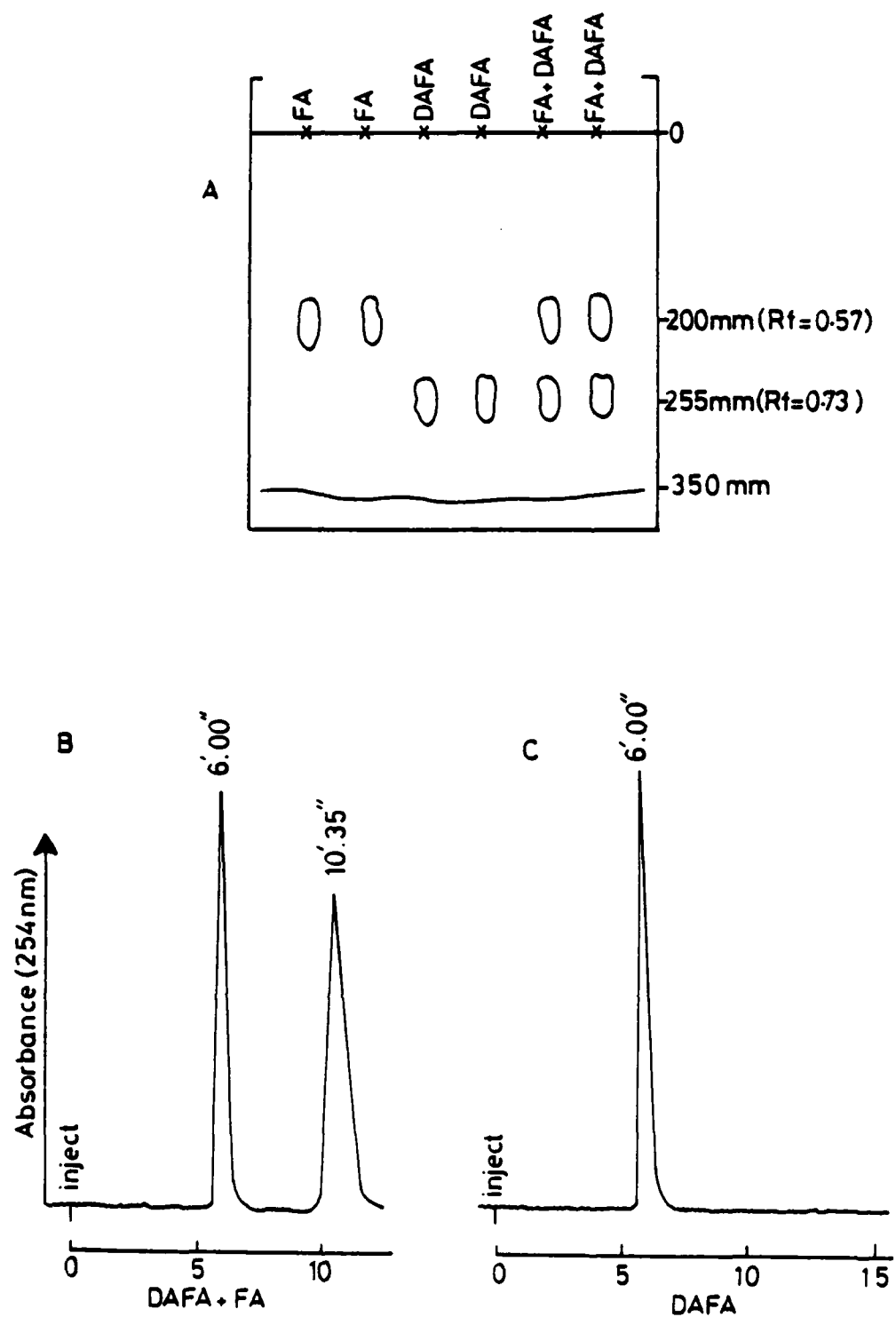


Fig 3.8

Figure 3.8    Paper Chromatography and HPLC of  
Synthesized DAFA

- (a) Folates (1mM) in 0.5%  $\text{Na}_2\text{CO}_3$  were applied as 50 $\mu\text{l}$  spots and the chromatogram developed for 3-3.5 hours with 0.5%  $\text{Na}_2\text{CO}_3$  as solvent.
- (b) HPLC was conducted on a Partsil 10/25 SAX Whatman column using an Altrex pump model 100A and an Altrex 165 variable wavelength detector. Flow rate of 50mM (Na/Na<sub>2</sub>)  $\text{PO}_4$  buffer (pH 7.0) was 1ml  $\text{min}^{-1}$ . Samples of 10 $\mu\text{l}$  containing 10 $\mu\text{g}$   $\text{ml}^{-1}$  of either folate (FA) or DAFA were injected. The abscissa is in minutes and the ordinate is 0.02 AUFS (absorption units full scale) at 254nm.

and a difference curve was obtained which compares well with a published curve (Bernstein et al., 1981). Comparison of the UV spectra for synthesized DAFA (figure 3.7c) with the DAFA spectra from folate deamination showed complete congruence.

Paper chromatography of DAFA (figure 3.8a) showed no detectable contamination with folate and further confirmed the identity of the synthesized DAFA; measured  $R_f$  values for folate and DAFA of 0.57 and 0.73, respectively, correspond well with past studies (Wurster and Butz, 1980).

The synthesized DAFA was recrystallized three times from HCl and then subjected to HPLC analysis. HPLC analysis (figure 3.8b) showed no detectable folate contamination (sensitivity >0.1%) in the DAFA; and the retention times for DAFA (6.0min) and folate (10.6min) were nearly identical to other reports (cf. Kakebeeke et al., 1980a), further confirming the authenticity of the synthesized product as DAFA. The recrystallized product was further purified by preparative paper chromatography before use in subsequent binding studies.

#### E Competitive Binding of Folate and DAFA to Cell Surface Receptors

Workers have suggested that the putative folate chemotactic receptor is detected only in the presence of excess DAFA (50 $\mu$ M) (De Wit, 1982). After numerous attempts to reproduce this result under a full spectrum of experimental conditions, I concluded that folate receptors insensitive to DAFA competition are not detectable by the assay reported by De Wit and used in this study (figure 3.9, Tillinghast and Newell, 1984). Others (Malchow, et al., personal communication) have since confirmed this finding and quite recently De Wit and Van Haastert (1985) have reported that they are unable to reproduce their earlier results. If the competition curves for [ $^3$ H]folate and folate (figure 3.3) or DAFA (figure 3.9a) are compared (see also Chapter 5),

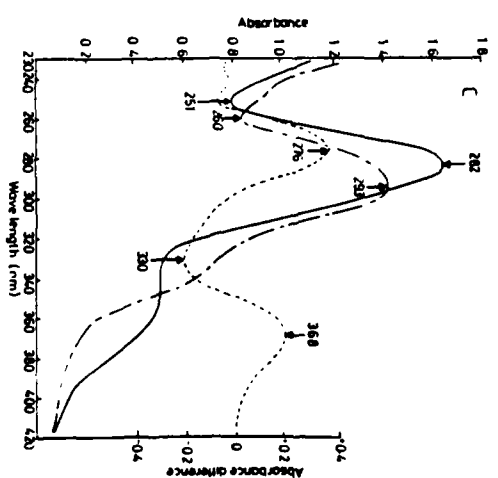
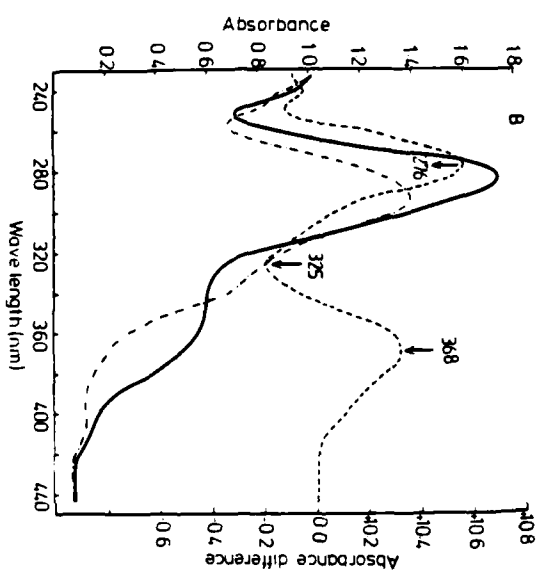
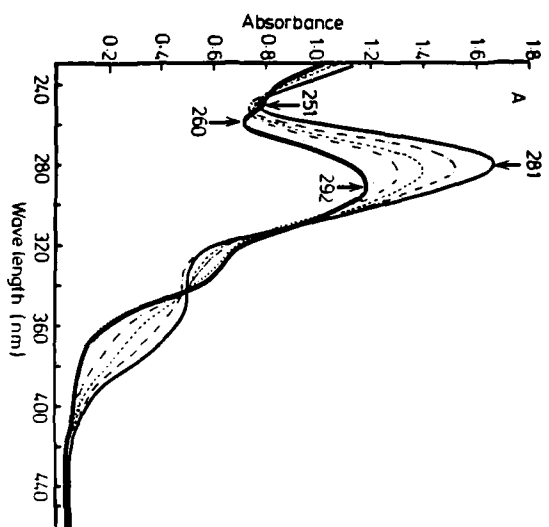


Fig 3 7

Figure 3.7    Comparison of Synthesized and  
Enzyme-produced DAFA

Folate and DAFA were prepared at 50uM in  
10mM PO<sub>4</sub> (K/K<sub>2</sub>) buffer pH 6.0.

(a) Folate was digested by an extracellular  
enzyme extract from D. lacteum and UV  
spectra recorded at

0 min (——)      5 min (— · — · —)  
15 min (— · — · —)      30 min (— · — · —)  
45 min (· · · · ·)      65 min (——)

after start of digestion.

(b) UV spectra of folate (——) and folate  
after 65min digestion (— · — · —) with D.  
lacteum enzyme extract. The difference  
curve (— · — · —) was obtained with folate  
in the sample cuvette and enzyme digest  
in the reference cuvette.

(c) UV spectra of folate (——) and  
synthesized DAFA (— · —). The difference  
curve (— · — · —) was obtained with folate  
in the sample cuvette and DAFA in the  
reference cuvette.

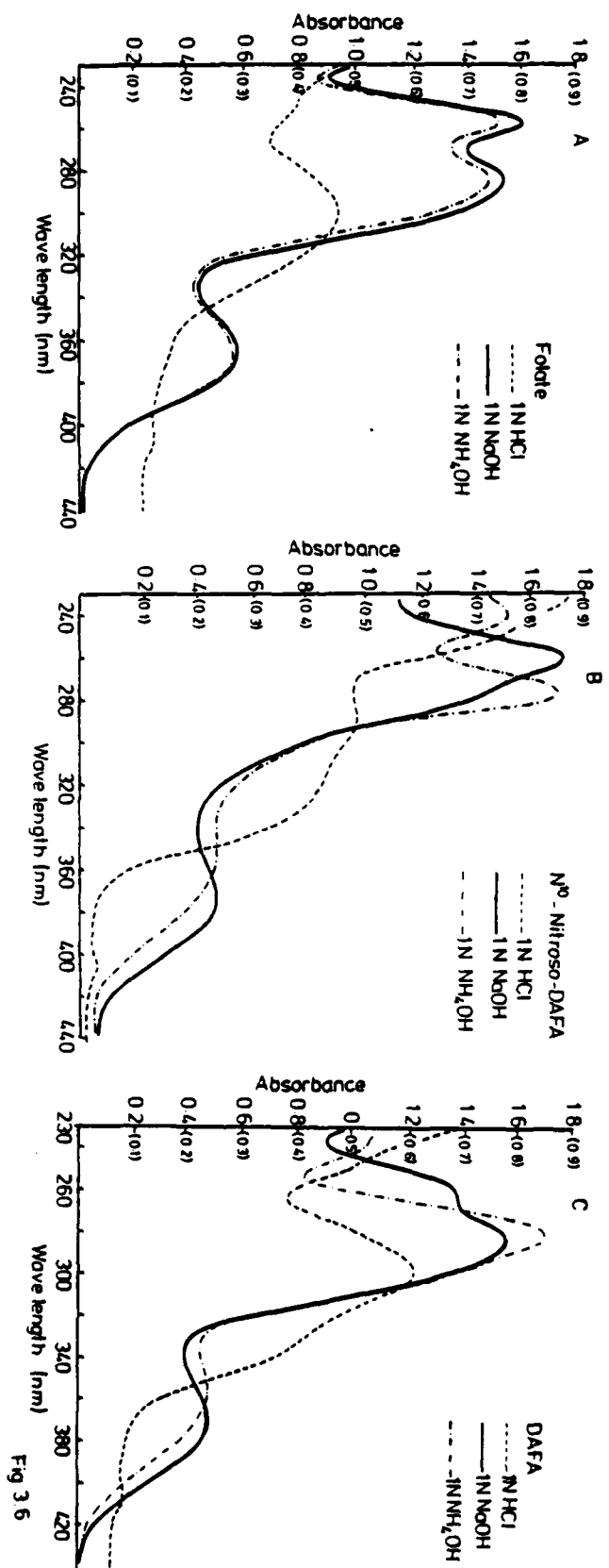


Fig 3.6

Figure 4.1

Cell-surface and Cytoskeletal Folate  
Receptors on NC4 through Development

$[^3\text{H}]$ folate binding to strain NC4 whole cells (—) and cytoskeletons (----) developed on filters (0-14hr) was measured at 1000nM ( $\Delta$ ), 500nM ( $\circ$ ), 200nM ( $\square$ ) and 50nM ( $\bullet$ ). Data for whole cells are means of three experiments and for cytoskeletons means of six experiments.

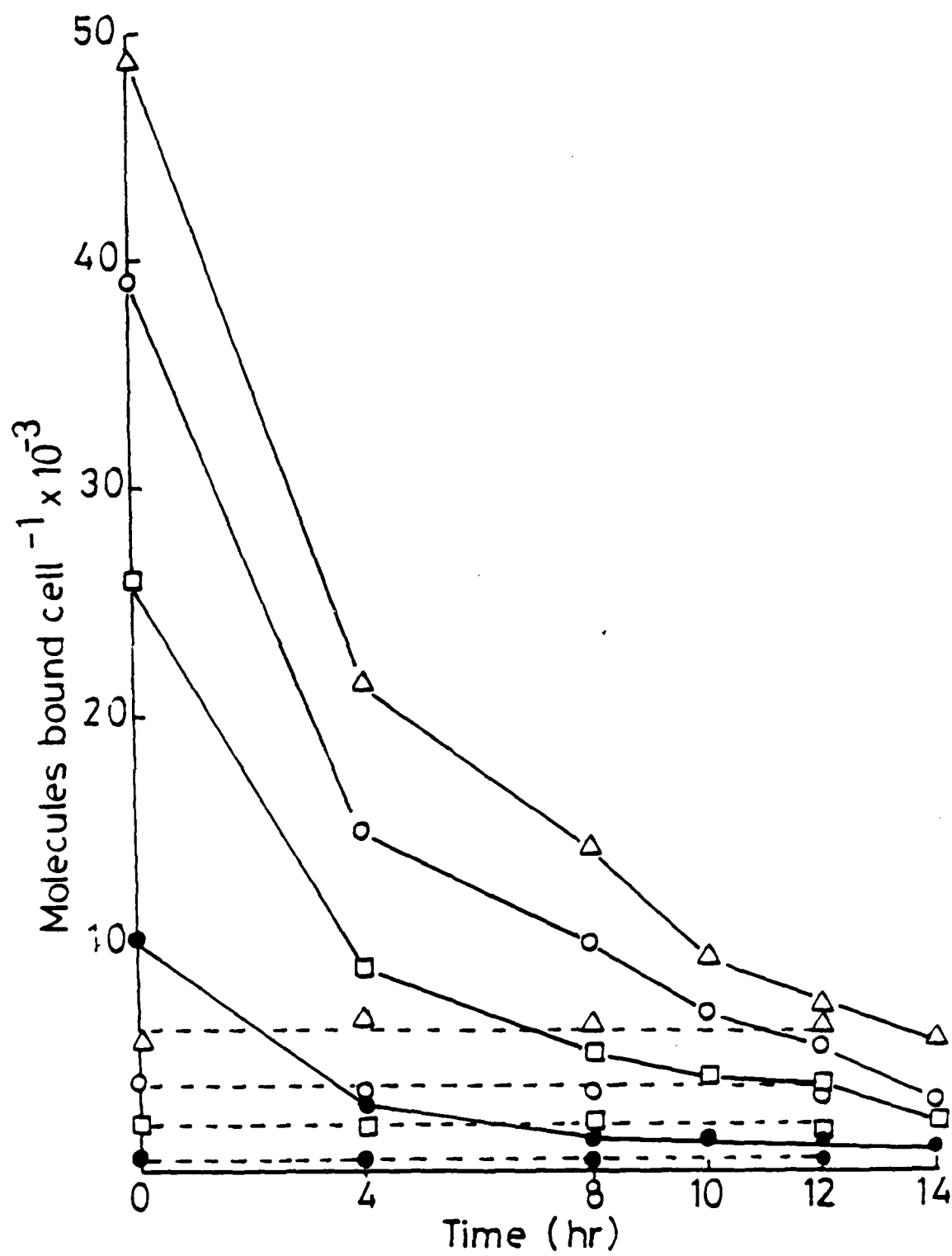


Fig. 4.1

Figure 4.2

Effect of 8hr Starvation on AX3 Folate  
Receptor Density

[<sup>3</sup>H]folate binding to whole cells, strain AX3, starved in shaker culture, was measured at 1000nM ( $\Delta$ ), 450nM ( $\bigcirc$ ), 250nM ( $\square$ ) and 50nM ( $\bullet$ ). This result is from a single experiment.

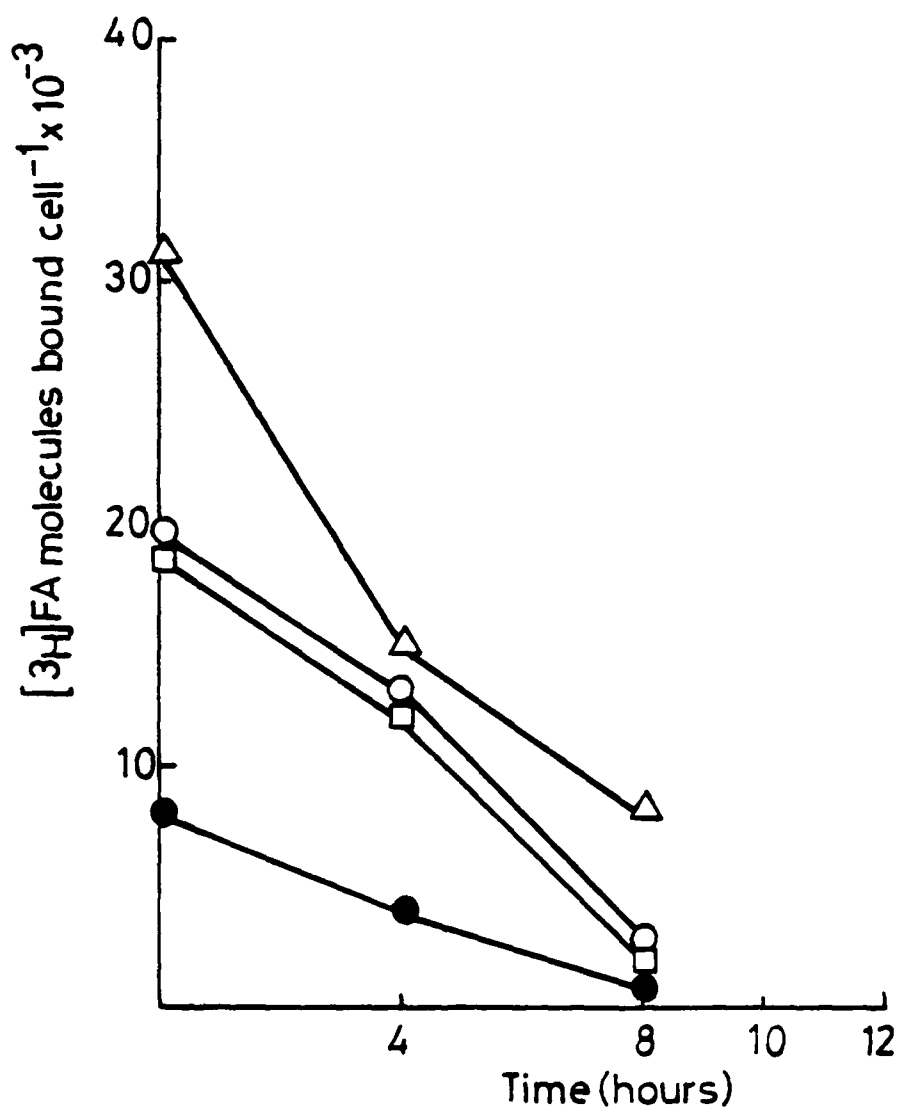


Fig 4. 2

binding to residual bacteria on the amoebal cell-surface and the reduced binding by 4hr was the result of phagocytosis of the bacteria. However, this theory was disproved by control binding studies with an axenic mutant; the mutant, AX3, when grown in axenic liquid media, showed a similar loss in receptor number (figure 4.2). As shown in figure 4.1, there was no reappearance of folate receptors at 12-14hr. However, there does appear to be a small pool of approximately 6000 receptors that are retained on the cell surface after the onset of starvation and development.

#### B Scatchard Plot Analysis of Folate Binding to Cytoskeletal Receptors

Vegetative whole cells were lysed with 1% Triton X-100 in the presence of EGTA and the deaminase inhibitor, 8-aza-guanine, as described in chapter 2 (Methods). The insoluble cytoskeletal proteins were allowed to bind [ $^3$ H]folate (30-1500nM) in competition with other soluble cell components and were then separated from the soluble components and unbound [ $^3$ H]folate by centrifugation through silicone oil. Scatchard plot analysis of data from eight experiments is shown in figure 4.3; analysis and line fit by least squares linear regression analysis showed 10,700 sites per cytoskeleton and a  $K_d$  of 470nM. The same data evaluated by distribution free plot analysis, using a microcomputer programme of Crabbe (1985), showed  $9900 \pm 880$  sites per cytoskeleton and a  $K_d$  of  $450 \pm 80$ nM (errors shown as S.E.M.). Control experiments of folate binding to whole cells were run in parallel with the cytoskeleton experiments using aliquots of the same cell suspensions and the same compositions of silicone separating oil and buffer as for the cytoskeleton assay. These conditions differ slightly from those described in chapter 3 for the whole cell assay. Scatchard plot analysis of the whole cells under these conditions showed 35,000 folate receptors per cell and a  $K_d$  of 210nM (figure 4.3, insert). Therefore, the

Figure 4.3

Scatchard Plot Analysis of Folate  
Binding to Cytoskeletons of Vegetative  
Amoebae

- a) Cytoskeletons were incubated with [ $^3$ H]folate (30-1500nM) in the presence of 8-aza-guanine (0.6mM) and non-specific binding controls additionally contained unlabelled folate (0.1mM). Data are means from eight independent experiments. Line fit was by least squares linear regression analysis and confirmed by distribution free plot analysis.
- b) Control binding to vegetative amoebae was assayed in the same buffer conditions and silicone oil as for cytoskeletons. Data are means from three separate experiments; line fit was by least squares linear regression analysis.

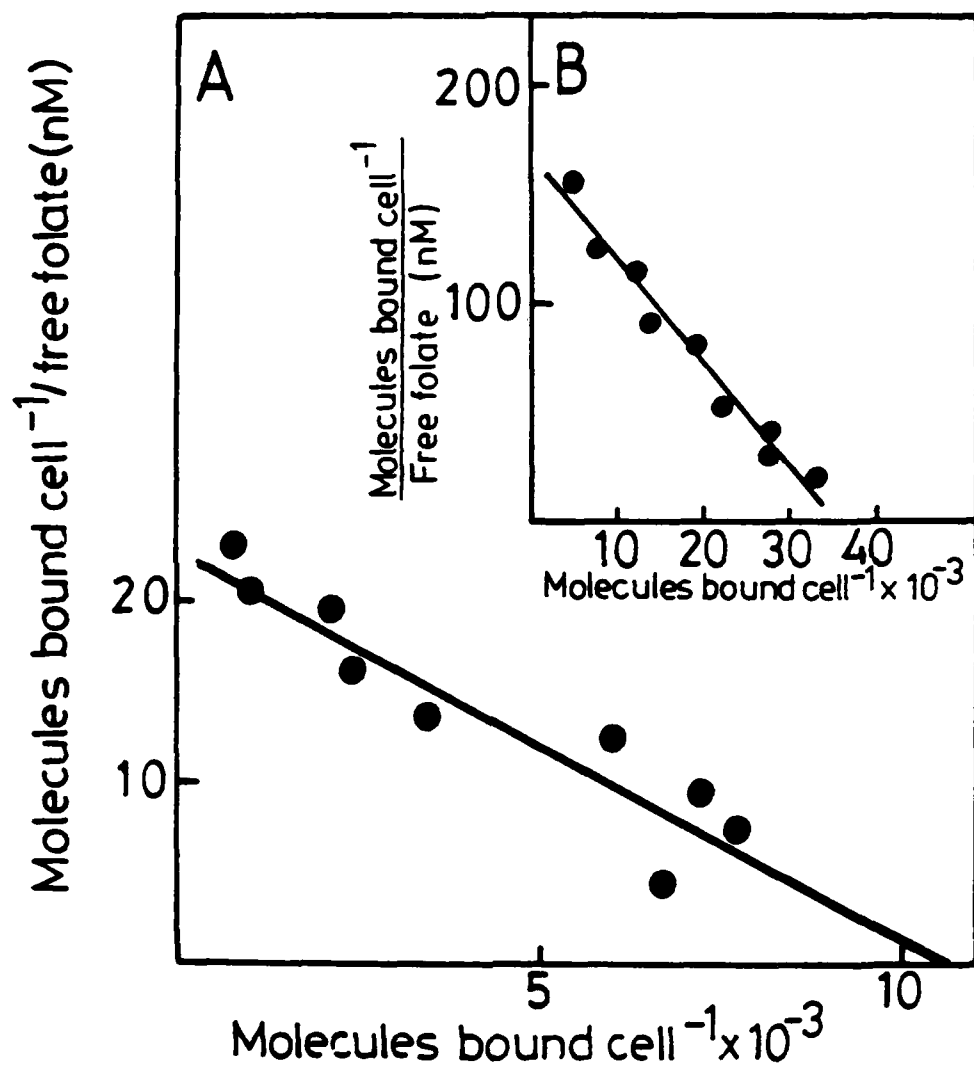


Fig 4.3

isolated vegetative cytoskeletons retain nearly 30% of the sites detected on intact amoebae.

#### C Cytoskeletal Folate Receptors through Development

The number of cytoskeletal folate receptors that were detectable during 12hr of development was about 6000 per cytoskeleton (figure 4.1), and it is noteworthy that this quantity is similar to the small pool of cell surface receptors that were found to be retained during the same period. It should also be pointed out that both of these numbers may be underestimated since the highest [ $^3\text{H}$ ]folate concentration used was 1000nM. Earlier data (chapter 3, figure 3.10) has shown that the receptors may not be fully saturated at folate concentrations below 1500nM. This may explain the difference in the number of sites found in this developmental regulation experiment when compared to the value of 10,000 reported in the Scatchard plot analysis (previous section).

#### 4.3 Discussion

My results indicate that the reappearance of folate responsiveness seen in the actin-accumulation studies does not involve reappearance of folate receptor sites later in development. The cell surface receptor density declines continually through development with only a residual pool of 6,000-10,000 receptors retained by 14hr of development. I demonstrated a similar sized pool of folate receptors on isolated cytoskeletons and found that this pool of receptors remain associated with the cytoskeleton from the vegetative stage through 12hr of development.

Although the folate receptor number did not increase later in development, the fact that roughly a third of the folate receptors are retained possibly suggests a role for folate in later development. Other workers have suggested such a role after finding that the enzyme folate deaminase continues to be produced in an active form after the

vegetative stage (Kakebeeke et al., 1980b). It has been shown that although folate elicits both chemokinetic (non-directional movement) and chemotactic (directional movement) cell responses in cells from the vegetative phase (Nandini-Kishore and Frazier, 1981; Varnum and Soll, 1981), the chemotactic cell response is lost by 6hr of development (Pan et al., 1972) while the chemokinetic response is retained through aggregation (Nandini-Kishore and Frazier, 1981; Varnum and Soll, 1981). The residual receptors that remain at 12-14hr may function in the chemokinetic mechanism. Alternatively, these residual receptors may play the primary role in chemotaxis throughout growth and may have a similar functional role in later development. It is then possible that the membrane surface receptors that are not associated with the cytoskeleton in vegetative amoebae may have no function in chemotactic movement but may play another role such as in folate transport (VanDriel, 1981) or as recently suggested, in stimulation of cAMP secretion (i.e. cAMP relay) (Devreotes, 1983; De Wit et al., 1985).

The association of folate receptors with the cytoskeleton is also intriguing. The direct association of ligand-binding receptors with the cytoskeleton or association through intermediate effector molecules has been suggested as a hypothesis to explain the heterogeneous binding characteristics of insulin (Jacobs and Cuatrecasas, 1976). This mobile receptor hypothesis continues to gain support. For example, cytoskeletal associations have more recently been demonstrated for the H-2 histocompatibility antigen (Koch and Smith, 1978), growth factor receptors (Schechter and Bothwell, 1981; Vale and Shooter, 1982), and N-formyl peptide chemotactic receptors on human granulocytes (Jesaitis et al., 1984). It is tempting to speculate that the residual folate receptors that remain on the surface of whole amoebae during development are those that are attached in some way to the cytoskeleton and it is

these same receptors that are then detected in the insoluble cytoskeleton extracts. Several of the studies cited above (Schechter and Bothwell, 1981; Vale and Shooter, 1982; Koch and Smith, 1978) have shown differential ligand affinities between the "free-receptors" and those associated with the cytoskeleton. They have also suggested that, in accordance with the mobile receptor hypothesis (Boeynaems and Dumont, 1975; Jacobs and Cuatrecasas, 1976), it is these cytoskeleton-linked receptors that actually transduce the ligand-initiated signal.

During the preparation of this thesis workers have reported variable affinity folate receptors and have identified several classes of receptors based on differential receptor kinetics (De Wit and Van Haastert, 1985; De Wit et al., 1985). De Wit and Bulgakov (1985) have suggested that the affinity of the folate receptors may be modulated through association with other membrane components. It seems, however, that the mobile receptor hypothesis provides a more elegant and equally feasible explanation of this data. I propose that the large pool of receptors not associated with the cytoskeleton may be a pool of low-affinity, fast-dissociating receptors as described by De Wit et al. (1985) and the cytoskeleton-associated pool of folate receptors are a high-affinity, slow-dissociating population linked to the chemotactic transduction mechanism. Granted, the low affinity  $K_d$  that I detected for the isolated cytoskeletons does not fit this hypothesis. However, it is possible that receptors detected on isolated cytoskeletons will demonstrate altered binding characteristics relative to receptors assayed while still associated with other membrane and cytoskeleton components. Similar reductions in folate receptor affinity have been shown on isolated membranes (De Wit and Bulgakov, 1985). This issue of cytoskeletal-mediated receptor modulation will be discussed in greater depth later in this thesis (chapter 7, general discussion).

## *Chapter 5*

## 5 THE SPECIFICITY OF THE FOLATE RECEPTOR EVALUATED BY COMPETITION BINDING STUDIES

### 5.1 Introduction

The specificity of the folate receptor has not previously been well characterized. These past studies have tested the binding of only a few competitive ligands and generally have used only a single concentration of the competitor (Wurster and Butz, 1980; Nandini-Kishore and Frazier, 1981; De Wit, 1982). Reports of folate derivatives that are more potent attractants than folate (Wurster and Butz, 1983) and the fact that rather high concentrations of folate ( $10^{-6}$ - $10^{-5}$ M) are required to induce folate-elicited responses such as cGMP synthesis (Van Haastert et al., 1981) and actin accumulation (McRobbie and Newell, 1983) lead me to suspect that folate might not be the primary ligand for the receptor. Additionally, the fact that DAFA binds to the receptor with an affinity equal to folate yet induces no chemotactic activity (De Wit, 1982; Van Haastert et al., 1982c; chapter 3, chapter 6) encouraged me to look for other molecules with such inconsistent characteristics in an attempt to further characterize this folate receptor. It also seemed that if a ligand of higher affinity was discovered this might provide a more sensitive assay for detecting subtle changes in folate receptor density that may occur through development. This chapter describes the results from a competitive binding assay used to test a full spectrum of folate derivatives for their ability to compete with [ $^3$ H]folate for folate receptor binding.

All derivatives were purchased in pure form or purified by preparative paper chromatography except N<sup>10</sup>-methyl-folate which was synthesized as described below.

In control studies, the concentration of folate required to inhibit binding of [ $^3$ H]folate (300nM) by 50% ( $I_{50}$ ) was also 300nM reflecting the

previously determined  $K_d$  for the folate receptor (chapter 3). Any competitor that shows an  $I_{50}$  of less than 300nM will have a greater affinity for the receptor than folate and any competitor with a  $I_{50}$  greater than 300nM will have a lower affinity for the receptor than folate.

The numbering sequence shown in figure 5.1 will be used when discussing various molecular substitutions to the folate molecule.

## 5.2 Results

### A Competitive Binding by Pterins

A selection of pterins were tested to determine the concentrations of these competitors required to inhibit [ $^3$ H]folate binding to the folate receptor (figure 5.2). Except for pteric acid, pterins with various group substitutions at the 6-position of the pteridine ring were unable to inhibit folate binding. The attachment of the complete p-aminobenzoic acid (p-ABA) moiety to the pteridine ring allowed pteric acid at high concentrations ( $I_{50}=10^{-5}$ M) to compete with folate for receptor binding. Pteric acid lacks the glutamate residue and consequently binds with an affinity 1/30th that of folate ( $I_{50}=9000$ nM).

### B Competitive Binding by Molecular Components of Folate

A variety of molecules which are fractional components of folate were evaluated for binding activity (figure 5.3). In spite of the fact that p-aminobenzoic acid (p-ABA) moiety greatly enhanced the binding of pterine to the receptor (i.e. pteric acid), p-ABA alone or when mixed with pterin-6-COOH showed no competitive ability. Pteric acid in the absence of the glutamate residue had reduced affinity relative to folate suggesting a significant contribution by glutamate. However, glutamate alone or when complexed with p-ABA, p-amino-benzoyl glutamate (p-ABA-glu), showed no binding inhibition. It has been reported that folates exist naturally with polyglutamate residues attached (Shane, 1980).

Figure 5.1     The Folate Molecule

Figure 5.7     Identification of  $N^{10}$ -methyl-folate

UV spectra of  $N^{10}$ -CH<sub>3</sub>-folate (-----) and methotrexate (——) and the difference curve (---) obtained with methotrexate in the sample cuvette and  $N^{10}$ -CH<sub>3</sub>-folate in the reference cuvette. Solvent was 1N HCl.

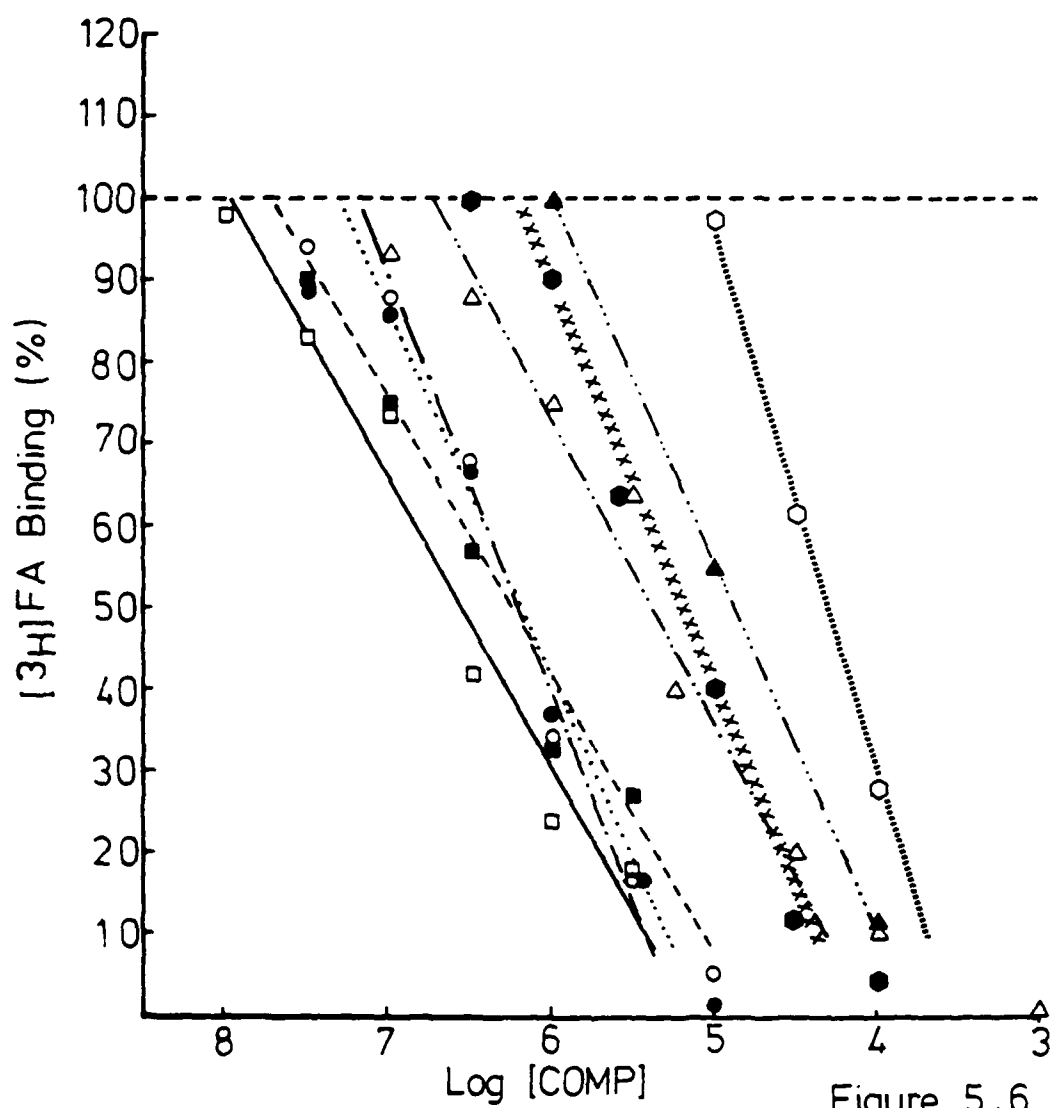


Figure 5.6

Figure 5.6      Composite of Competitive Binding Curves for  
Effective Folate Competitors

This composite figure allows comparison of the ligand concentrations required to produce half-maximal inhibition ( $I_{50}$ ) of [ $^3H$ ]folate binding. Data for the following ligands were consolidated from figures 5.2-5.5.

Aminopterin	(——)
Methotrexate (MTX)	(----)
Folate	(—.—)
DAFA	(.....)
$N^{10}$ -CH <sub>3</sub> -FA	(—...—)
$N^{10}$ -nitroso-DAFA	(XXXXXX)
Dichloro-MTX	(—...—)
Pteronic Acid	(.....)

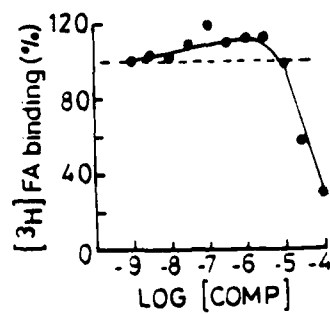
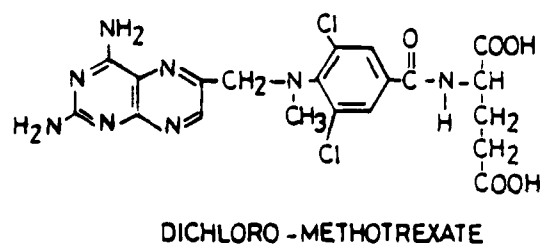
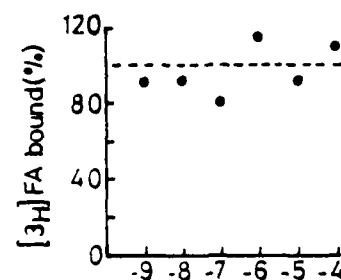
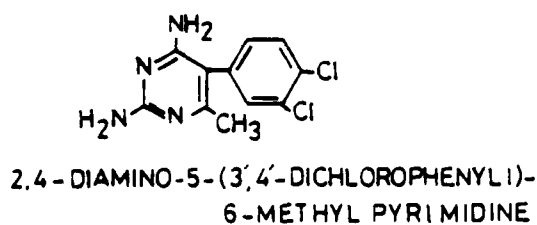
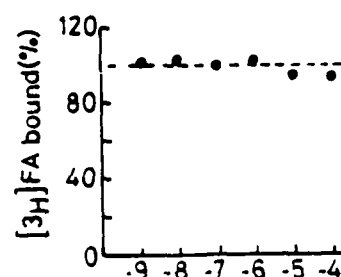
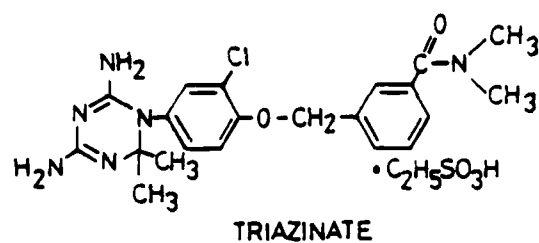


Fig. 5. 5

Figure 5.5     Competitive Binding of Anti-neoplastic Folate  
Analogues with [<sup>3</sup>H]folate

Vegetative amoebae were incubated with [<sup>3</sup>H]folate (300nM), various concentrations of folate analogues ( $10^{-9}$ - $10^{-4}$ ) and 8-azaguanine (0.5mM). Data for each analogue are means from two experiments.

binding as reflected in the reduced affinities of these analogues (figures 5.4, 5.6). The larger electronegative nitroso-group showed greater disruption of receptor association than the methyl substitution, emphasizing the need for direct receptor-ligand interaction at the N<sup>10</sup>-position.

#### D Competitive Binding of Other Anti-neoplastic Folate Analogues

Many anti-neoplastic folate analogues are effective inhibitors of the cytoplasmic enzyme, dihydrofolate reductase (DHFR) and thereby disrupt thymidine biosynthesis (Jackson, 1978; Jackson, 1984). Three such analogues were evaluated for folate receptor binding. Two non-classical folate analogues, triazinate and metoprine (2,4-diamino-(3',4'-dichlorophenyl)-6-methyl pyrimidine (DDMP)), showed no binding to the folate receptor (figure 5.5). The classical folate analogue, dichloromethotrexate, used selectively in liver tumour therapy, showed some binding activity. However, the chlorine substitutions on the benzene ring of p-ABA seriously hindered receptor-ligand interactions in this region.

The relative binding activities of the effective competitors are compared in the composite diagram which was reconstructed from the individual competition curves (figure 5.6).

#### 5.3 Discussion

No ligands were discovered that possess significantly higher affinity for the folate receptor. Other workers (Wurster and Butz, 1983) have demonstrated light-scattering responses with D. discoideum induced by only 0.1nM N<sup>10</sup>-methyl-folate whereas, they found that folate did not elicit a response at concentrations below 10nM; yet, I found in this study that the binding affinity of N<sup>10</sup>-methyl-folate was less than folate (I<sub>50</sub>=3000nM) (figure 5.6, see further discussion, chapter 7).

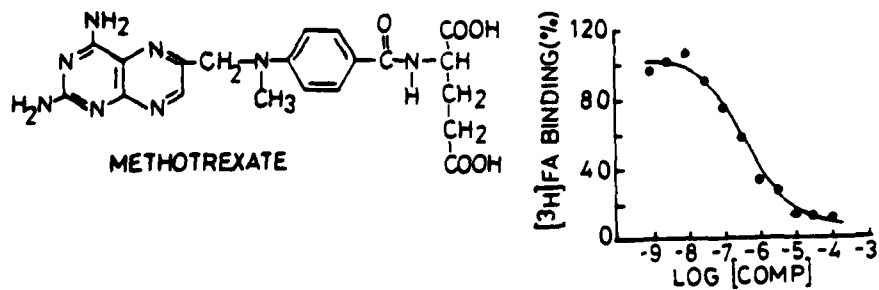
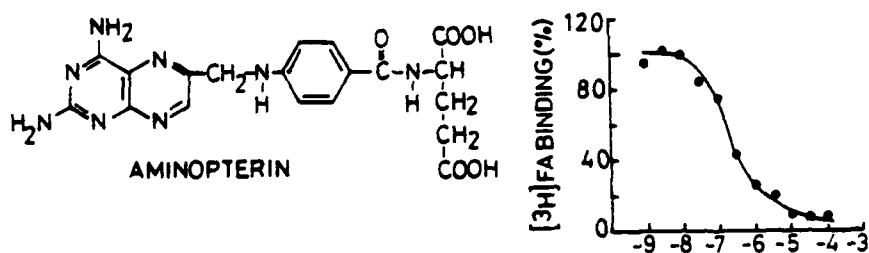
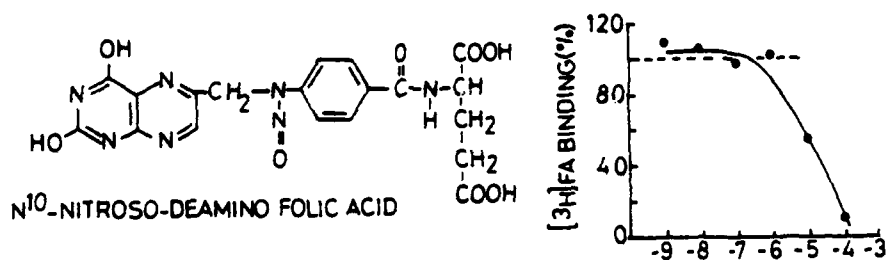
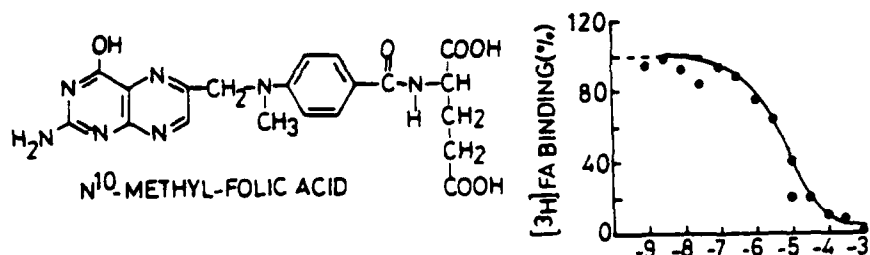
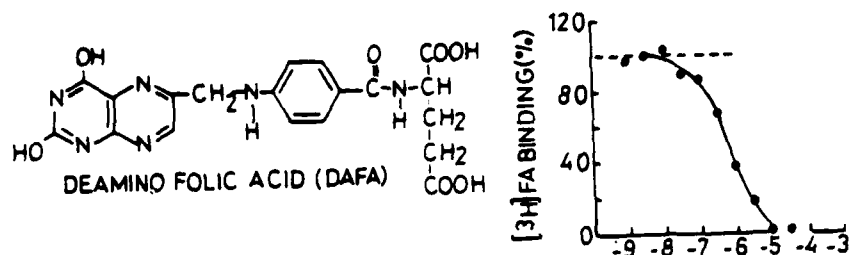
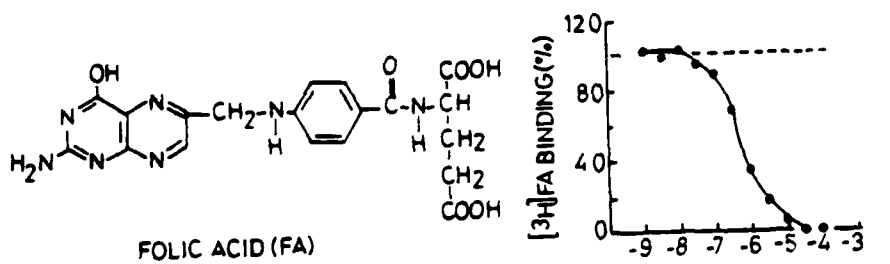


FIG. 5.4

Figure 5.4     Competitive Binding of Folate Analogues with  
[<sup>3</sup>H]folate

Vegetative amoebae were incubated with [<sup>3</sup>H]folate (300nM) and various concentrations of folate analogues ( $10^{-9}$ - $10^{-4}$ ), and 8-aza-guanine (0.5mM). Data for each analogue are means from at least two experiments.

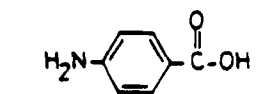
This lead me to test the binding of polyglutamate. Polyglutamate showed no binding activity, although, a tail of 50 residues is no doubt excessive. By testing these molecular components, it became evident that, as suggested for chemotaxis (Pan et al., 1975), the pteridine ring was essential for binding activity; but, as shown in section C, ligand binding is optimal only when all molecular components are present and covalently linked.

### C Competitive Binding by Classical Folates

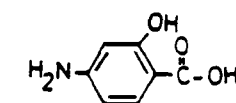
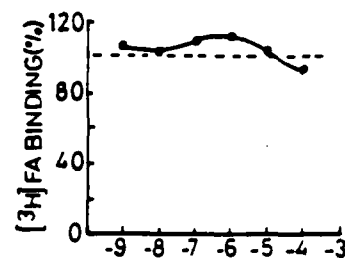
All the classical folates, possessing the general folate backbone but with minor group substitutions, showed strong competitive binding activities (figure 5.4). The 2,4-diamino-folates, aminopterin and methotrexate (MTX, amethopterin), known to be effective anti-neoplastic agents (for review, Jackson, 1984), showed binding affinities somewhat higher than folate (figure 5.6). The replacement of the -OH with an -NH<sub>2</sub> at position 4 on the pteridine ring apparently enhances, or at least has no negative effect on, ligand binding to the receptor. DAFA, as shown previously (chapter 3), binds with an affinity equal to folate, reflecting again the insensitive nature of the receptor to minor pteridine ring alterations.

Because of reports of enhanced chemotactic response to N<sup>10</sup>-methyl-folate (Wurster and Butz, 1983), I synthesized N<sup>10</sup>-methyl-folate from methotrexate by anaerobic alkaline hydrolysis of the C<sup>4</sup>-amino group (Cosulich and Smith, 1948) and identified the product by both UV spectroscopy (figure 5.7) and paper chromatography (Wurster and Butz, 1983). Purified N<sup>10</sup>-methyl-folate was obtained after preparative paper chromatography (chapter 2,3).

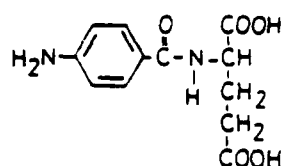
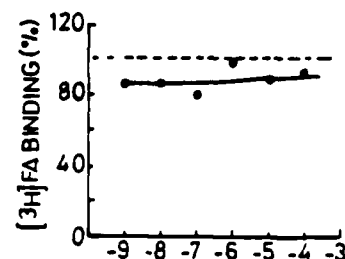
The methyl-substitution at the N<sup>10</sup>-position of folate or substitution with a nitroso-moiety on DAFA (ref. chapter 3 for synthesis) seems to cause at least minor steric hindrance to receptor



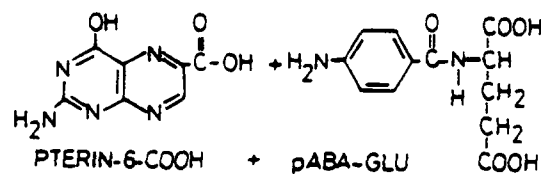
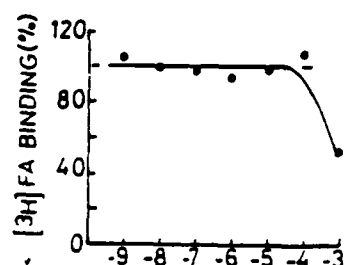
p-AMINO BENZOIC ACID



p-AMINOSALICYLIC ACID

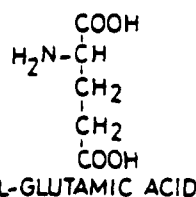
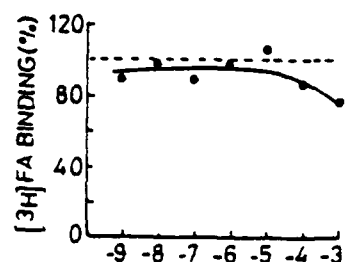
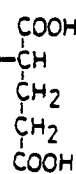


p-AMINO BENZOYL-GLUTAMIC ACID

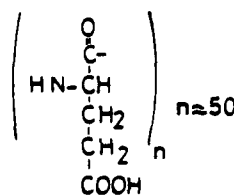
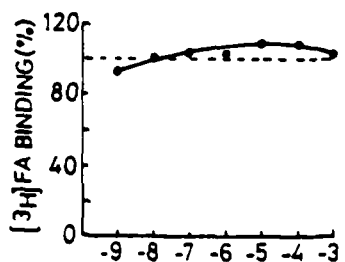


PTERIN-6-COOH

+ pABA-GLU



L-GLUTAMIC ACID



POLY-L-GLUTAMIC ACID

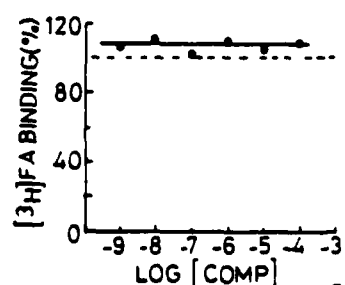


FIG. 5.3

Figure 5.3      Competitive Binding of the Molecular  
Components of Folate with [<sup>3</sup>H]folate

Vegetative amoebae were incubated with [<sup>3</sup>H]folate (300nM), various concentrations of molecular components ( $10^{-9}$ - $10^{-4}$ ) and 8-azaguanine (0.5mM). Data for each component are means from two experiments.

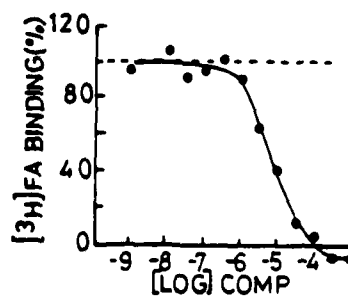
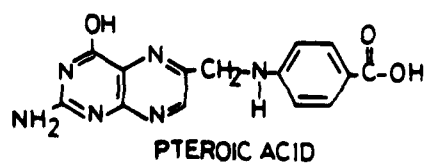
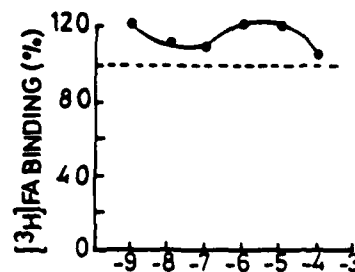
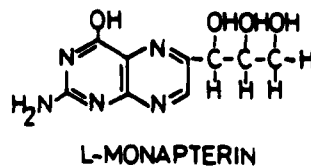
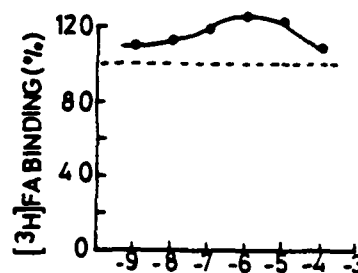
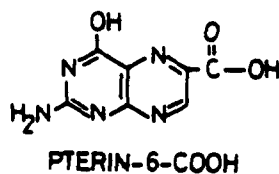
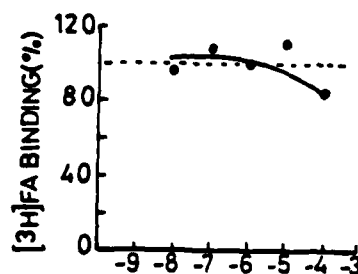
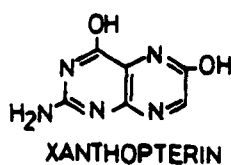
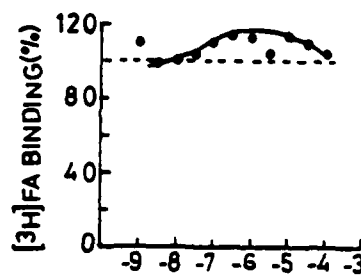
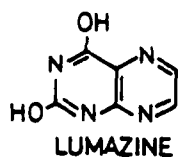
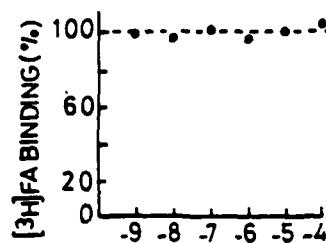
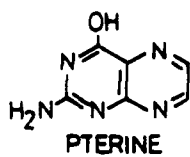


FIG 5.2

Figure 5.2

Competitive Binding of Pterins and [<sup>3</sup>H]folate

Vegetative amoebae were incubated with [<sup>3</sup>H]folate (300nM), various concentrations of pterins ( $10^{-9}$ - $10^{-4}$ ) and 8-aza-guanine (0.5mM). Data for each pterin are means from two experiments.

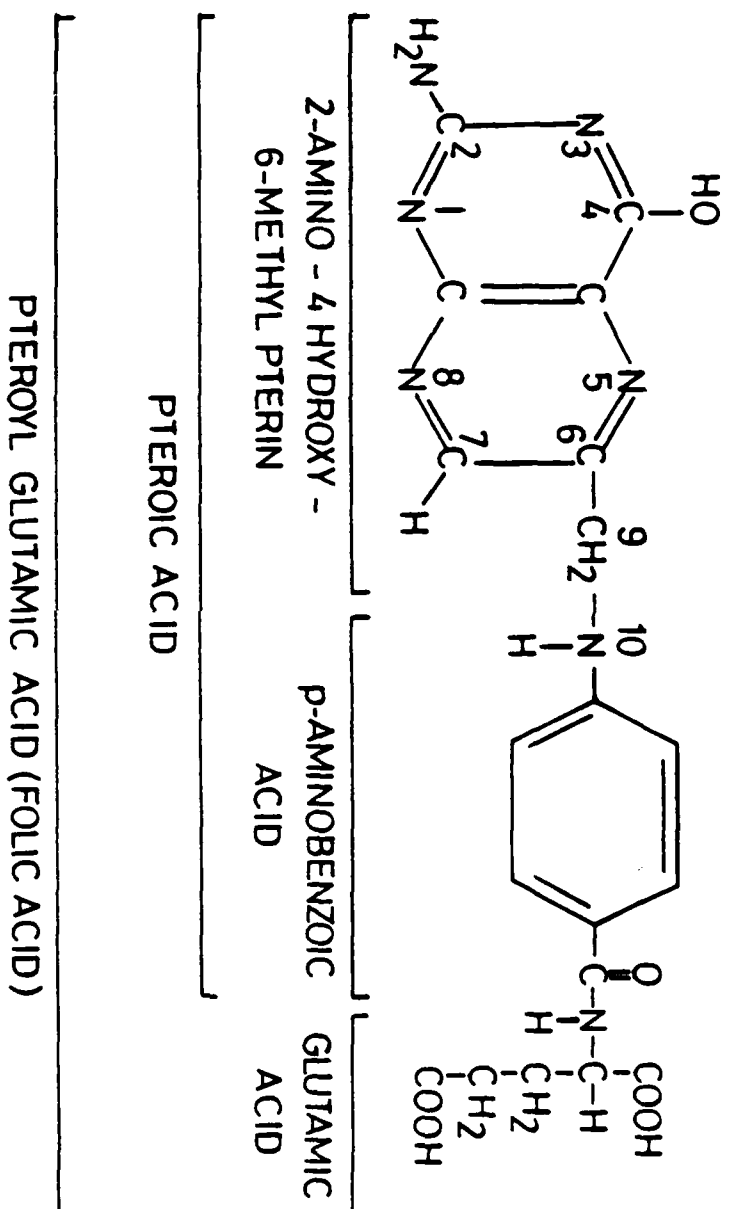


Fig. 5.1

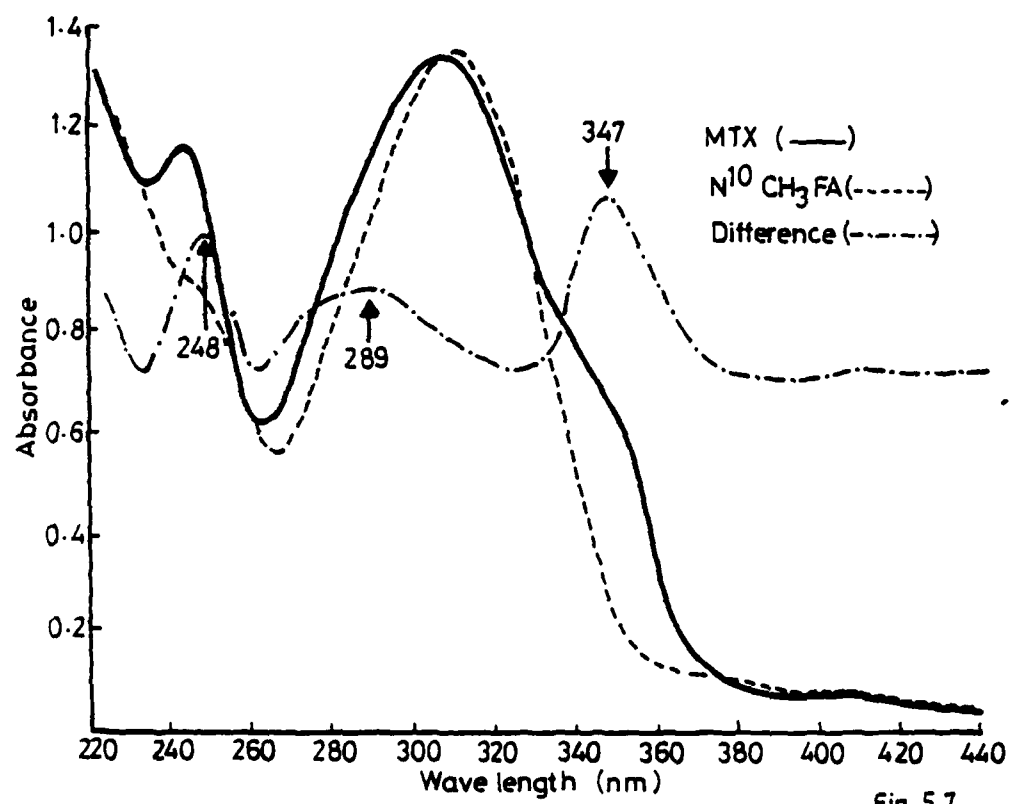


Fig 5.7

Another non-congruent situation might exist for methotrexate. Although Nandini-Kishore and Frazier, (1981) have shown methotrexate to be a suitable chemoattractant for D. discoideum, others have found that methotrexate is not chemotactic and have suggested that methotrexate samples in the earlier study were contaminated with N<sup>10</sup>-methyl-folate (Wurster and Butz, 1983). If methotrexate is not chemotactic (see also chapter 6), then the strong binding of methotrexate would be an inconsistency; it is, however, clearly possible to postulate ligands that bind but do not elicit a response.

From this competitive binding data, it was possible to make several judgements about the specificity of the folate receptor. The only ligands that bound effectively were those that contained the entire folate moiety (except in the case of pteric acid which is missing glutamate). Minor exchanges of hydroxyl (-OH) and amino (-NH<sub>2</sub>) groups on the pteridine ring showed no hindrance to binding (e.g. methotrexate, aminopterin, DAFA). The folate receptor showed sensitivity to ligand substitutions on the N<sup>10</sup>-position (N<sup>10</sup>-methyl-folate, N<sup>10</sup>-nitroso-DAFA) and also to substitutions on the benzene ring of p-ABA (dichloromethotrexate). Even though pteric acid showed binding activity despite the absence of the glutamate moiety, this terminal moiety obviously enhances receptor binding and therefore a degree of interaction between the receptor and the glutamate must occur. A possible folate receptor configuration is schematically shown in figure 5.8, emphasizing the sensitivity to N<sup>10</sup> substitutions and to benzene ring substitutions, and the importance of glutamate.

Figure 5.8     Schematic Drawing of the Folate Receptor

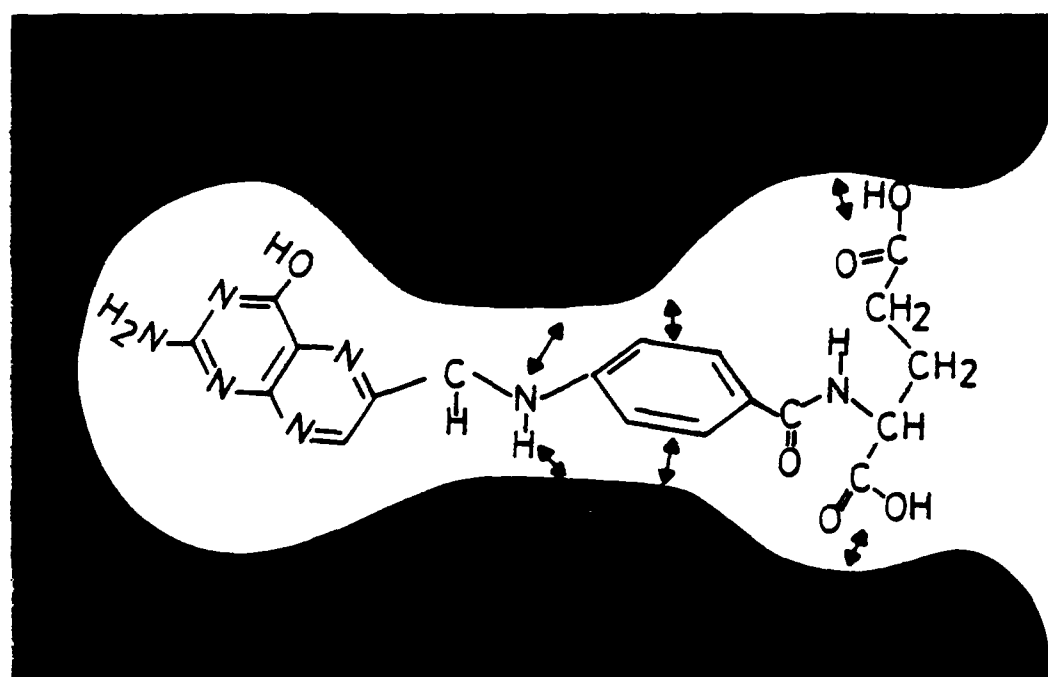


Fig. 5.8

## *Chapter 6*

## 6 THE CHEMOTACTIC RESPONSE TO FOLATES AND PTERIDINES BY VEGETATIVE AND DEVELOPING CELLS

### 6.1 Introduction

Folate analogues that bind to the folate receptor with an affinity comparable to folate have met only one criterion that would allow classification of these molecules as chemoattractants. Additionally, these molecules must elicit receptor-mediated cellular responses. The fact that a receptor binds a ligand does not necessitate that the receptor will in turn transduce the chemoattractant signal. A receptor-mediated cellular response that can be observed is chemotaxis itself. Chemotactic assays allowed me to correlate the binding characteristics of a variety of ligands with the induced chemotactic response in vegetative and developing cells.

Chemotactic assays applied to Dictyostelium discoideum in past studies include, the cellophane square test (Bonner et al., 1966), the small population assay (Konijn, 1970) and a variety of assays that allow migration on agar surfaces (Nandini-Kishore and Frazier, 1981; Varum and Soll, 1981). Several of these assays expose the test cells to a uniform concentration of chemoattractant (Bonner et al., 1966; Varum and Soll, 1981), while others create an artificial chemoattractant gradient and a directional stimulus by localizing the applied attractant on only one side of the test cell population (Konijn, 1970; Varum and Soll, 1981). The tests which bathe the cells in a uniform concentration of chemoattractant may test the chemical's potential to stimulate rate of movement but may not show whether the attractant possesses the potential to orientate moving cells (Bonner et al., 1966) unless a gradient is established by cellular enzymes. Alternatively, the tests that utilize an artificial, directional gradient of chemoattractant test both rate stimulation and cell orientation. An exception to this may be

the small population assay on hydrophobic agar (Konijn, 1970); this directional test may only successfully reflect the ability of a chemical to induce cell orientation since the test cells are restricted within the liquid droplet on the agar and rate of movement is not easily assessed.

Limited scale chemotactic response studies have been conducted as reviewed above but in most cases detailed correlations with receptor binding data have not been possible. In this chapter, I present results from the non-directional "spreading" assay and the "directional" assay and correlate these data with ligand binding affinities.

## 6.2 RESULTS AND DISCUSSION

Movement in either chemotactic assay method was evident after only 1-2hr of development; however, I found it equally valid, and the evidence less ambiguous, if the degree of chemotactic migration was recorded photographically using darkfield optics after 4hr of migration for the "directional" assay and 7-12hr for the "spreading" assay. A portion of the cell population in the central zone where the chemoattractant is fully degraded often demonstrated the developmental changes expected when cells are starved for 7-12hr on agar. However, those cells at the migration front seemed to migrate indefinitely and to maintain a unicellular state, suggesting that cell-contact inhibition continues to occur (Samuel, 1961). By monitoring cell migration through 12hr on the "spreading" assay, it was possible to detect the onset of chemoattractant sensitivity as the cells proceeded through development in the central zone.

### A Chemotactic Response Toward Pterin Analogues Evaluated by the "Spreading" Assay

The pterins: lumazine, xanthopterin, pterine-6-COOH, L-monapterin and pteric acid were tested with the "spreading" assay for their

ability to induce a chemotactic response in vegetative amoebae of wild-type strain NC4 (figure 6.1). Only pterin-6-COOH and pteronic acid elicited chemotactic activity. The response elicited by pterin-6-COOH was unexpected as the binding data (chapter 5) for this pterin showed no inhibition of [ $^3$ H]folate binding. This data may, however, indicate the presence of a separate pterin receptor which binds only pterins and elicits its own chemotactic response as reported by others (Van Haastert et al., 1982c). Lack of a response with either lumazine or xanthopterin may reflect a high degree of selectivity by this putative pterin receptor. The chemotactic response seen for pteronic acid (figure 6.1) is consistent with the binding data as it was shown that pteronic acid inhibited folate binding (figure 5.2). It, therefore, seems likely that this response is mediated by the folate receptor, even though pteronic acid binds with an apparent affinity only 1/30th that of folate (chapter 5). It is also possible that pteronic acid binds to the pterin receptor which in turn induces chemotaxis.

#### B Chemotactic Response Toward Folate Analogues Evaluated by the "Spreading" Assay

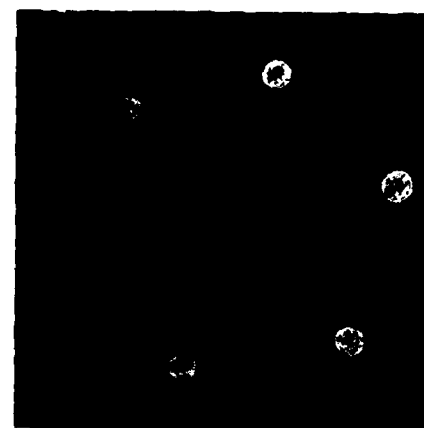
All folates tested had previously shown at least some ability to inhibit folate binding to its receptor (chapter 5). However, these folates induced a wide spectrum of chemotactic responses (figure 5.2). DAFA and N<sup>10</sup>-nitroso-DAFA elicited no response from vegetative amoebae thus confirming previous reports for D. discoideum (Pan and Wurster, 1978; Van Haastert et al., 1982c). Vegetative cells responded positively to both methotrexate and aminopterin (figure 6.2); however, the characteristics of the cell migration pattern were distinctly different from the folate response (figure 6.2). The migration pattern of cells stimulated by folate showed a distinct, dense leading edge whereas, the migration patterns from aminopterin and methotrexate tests showed more

Figure 6.1     "Spreading" Chemotactic Response of  
Vegetative Amoebae to Pterins

Vegetative amoebae of strain NC4, were tested for their chemotactic response to various pterins ( $10^{-5}M$ ) added to SORPHOS buffered agar. The "spreading" response was recorded after 7hr migration by photography through darkfield optics (magnification = 1.5X)



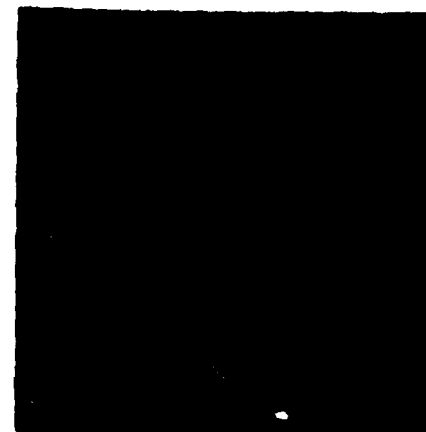
Buffered agar



Lumazine  $10^{-5}M$



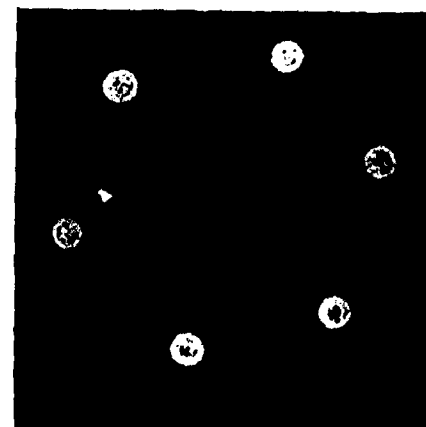
L-Monapterin  $10^{-5}M$



Xanthopterin  $10^{-5}M$



Pterin-6-COOH  $10^{-5}M$



Pteroic acid  $10^{-5}M$

Fig 6.1

Figure 6.2     "Spreading" Chemotactic Response of  
Vegetative Amoebae to Folate Analogues

Vegetative amoebae of strain NC4 were tested for their chemotactic response to various folates ( $10^{-5}$ M) added to SORPHOS buffered agar. The "spreading" response was recorded after 7hr migration by photography through darkfield optics (magnification = 1.5X).

diffuse, evenly-dispersed cell populations. A potential explanation is that methotrexate and aminopterin induce an increase in the rate of cell migration but do not provide a means for cell orientation (i.e., they acted as chemokinetic rather than chemotactic agents). To test this hypothesis, these same folates were evaluated by the "directional" chemotaxis assay.

#### C "Directional" Chemotactic Response Toward Folates

This assay was conducted on a microscope slide with the test cells dotted between two narrow troughs cut in the agar (details in chapter 2). The chemoattractant being tested was placed in one trough and SORPHOS buffer in the opposite trough. Cell migration was recorded photographically as before. An obvious preferential migration of cells toward the trough containing the test chemical was evidence that this chemical could induce orientated migration.

Folate, N<sup>10</sup>-methyl-folate and pteric acid elicited strong preferential migration toward the chemoattractant trough (figure 6.3). DAFA showed a migration pattern identical to the buffer control. The migration patterns for methotrexate and aminopterin were slightly skewed in favour of the chemoattractant trough; but, again, there was no distinct leading edge of migrating cells as seen for folate, pteric acid and N<sup>10</sup>-methyl-folate. Though not truly definitive, these results suggest that, as hypothesized, methotrexate and aminopterin stimulate the rate of cell migration but provide an improper signal for cell orientation.

As noted with the "spreading" assay, there is a distinct disparity between the receptor binding characteristics of the DAFA analogues, and possibly methotrexate and aminopterin, and their ability to elicit a chemotactic response. As suggested by De Wit and Van Haastert (1985), either the folate binding assay employed in chapters 4 and 5 does not

Figure 6.3     "Directional" Chemotactic Response of  
Vegetative Amoebae toward Folate Analogues

Vegetative amoebae of strain NC4 were tested for "directional" chemotactic response to various folates. The chemoattractant ( $10^{-4}$  M) was placed in the designated trough and SORPHOS in the other trough. These photographs (through darkfield optics) were taken after 4hr migration and are representative examples from two separate experiments.

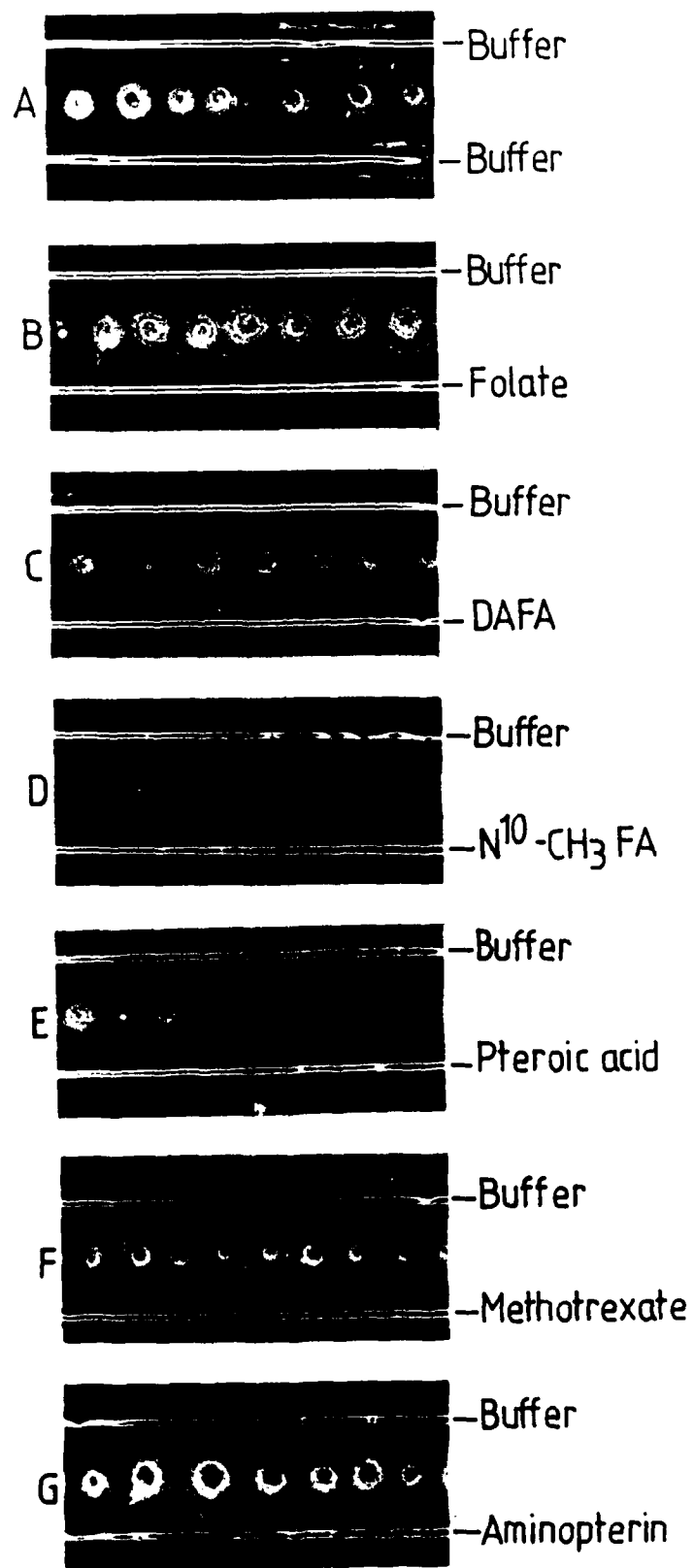


Fig 6.3

other sites. This competition assay showed an  $I_{50}$  of 30nM for folate competition with [ $^3$ H]folate; this value correlates well with the 17nM  $K_d$  reported by De Wit et al. (1985) for B-sites and is a 10-fold lower  $K_d$  than that seen for the major receptor population ( $K_d$ =300nM, chapter 3). The maximum binding measured in two separate experiments revealed 165 B-sites per cell which is significantly lower than the density of 550 sites per cell reported by De Wit.

#### B Competitive Inhibition of [ $^3$ H]folate Binding to B-sites

Preliminary competitive binding data with two other ligands, methotrexate and DAFA, also showed inhibition curves comparable to the earlier B-site study (figure 7.2). However, in competition with 10nM [ $^3$ H]folate, methotrexate demonstrated an  $I_{50}$  of 2000nM which reflected an affinity 10-fold higher than that reported by De Wit who found an  $I_{50}$  of 20,000 (figure 7.1). This was a single experiment and may therefore reflect experimental error. Alternatively, if the methotrexate used had been partially degraded to  $N^{10}$ -CH<sub>3</sub>-folate, then a significantly altered binding inhibition would be predicted since  $N^{10}$ -CH<sub>3</sub>-folate binds to the B-sites with a very high affinity ( $K_d$ =4nM). L-monapterin, shown to induce chemotaxis late in development (12-24hr, chapter 6), showed no inhibition of [ $^3$ H]folate binding to B-sites of vegetative amoebae (figure 7.2).

#### C Developmental Regulation of B-sites

The B-site assay was also employed to detect these receptors through development. Table 7.2 summarizes data from a single experiment which showed that B-sites were present later in development and suggested a very slight increase in the density of receptors at 10hr of development. More definitively, these results also show that [ $^3$ H]folate binding to B-sites is not sensitive to L-monapterin inhibition at 8, 10

Table 7.2

Binding Calculations	<u>B-SITES THROUGH DEVELOPMENT</u> [ <sup>3</sup> H]folate Molecules Bound Cell <sup>-1</sup>		
	8 hours	10 hours	12hours
Total bound folate after dissociation	198	270	230
Non-specific bound with excess folate present	84	73	103
Folate bound specifically	114	197	127
Folate bound with L-monapterin present	192	226	215

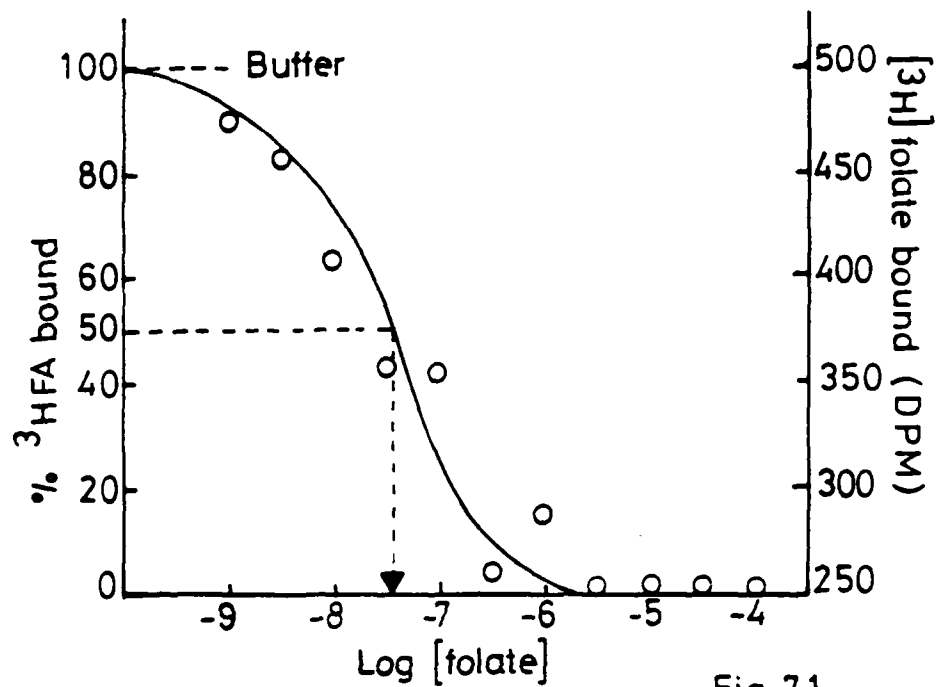


Fig. 7.1

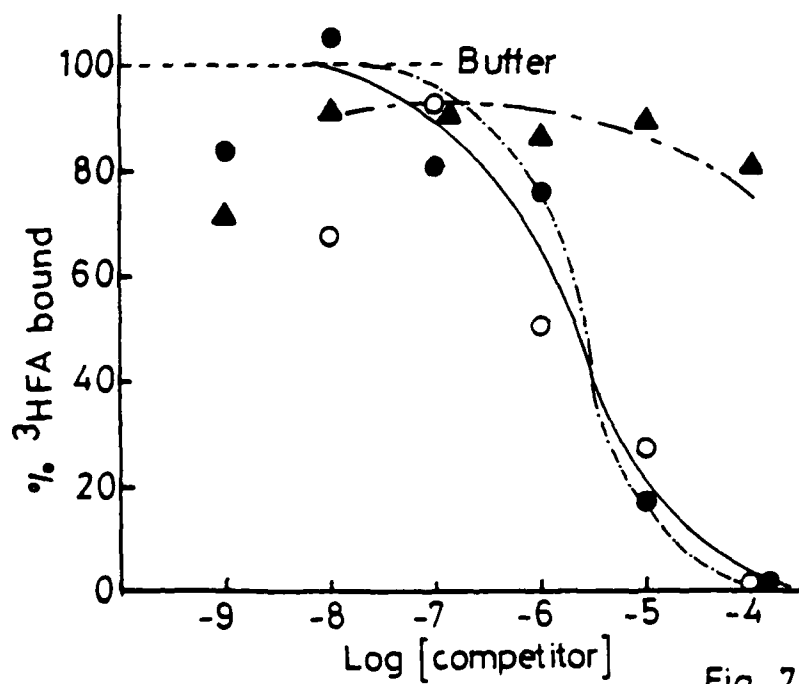


Fig. 7.2

Figure 7.1 [<sup>3</sup>H]folate Binding to B-sites

Vegetative cells were incubated with [<sup>3</sup>H]folate (10nM), 8-aza-guanine (0.33mM), and various concentrations of unlabelled folate ( $10^{-9}$ - $10^{-4}$ M) for 120sec. Cells were then exposed to a dissociation solution of unlabelled folate (25μM) for 60sec and finally recovered by centrifugation through silicone oil. The buffer line (----) shows [<sup>3</sup>H]folate binding in the presence of SORPHOS buffer in place of the folate competitor. I<sub>50</sub> is shown by the arrowed, dashed line. This was a single experiment.

Figure 7.2 Competitive Binding to B-sites

Vegetative cells were incubated with [<sup>3</sup>H]folate (10nM), 8-aza-guanine (0.33mM), and various concentrations ( $10^{-9}$ - $10^{-4}$ M) of the competitors methotrexate (●), DAFA (○), and L-monapterin (▲) for 120sec. Cells were then exposed to a dissociation solution (25uM folate) for 60sec and finally recovered by centrifugation through silicone oil. The buffer line (----) shows [<sup>3</sup>H]folate binding in the presence of SORPHOS buffer in place of the competitor. These data are from a single experiment.

binding characteristics similar to those previously reported? c) Are B-sites developmentally regulated (i.e., Are B-sites present at 12hr of development and could they function in the 12hr actin-accumulation response?)?

The data presented in this chapter is quite preliminary and consequently the main focus of the chapter is not the results. This chapter serves to introduce in some detail the characteristics of the A-sites, B-sites, and C-sites as reported and to discuss these characteristics in light of the binding and chemotaxis data previously presented in this thesis.

## 7.2 Results

### A Slow Dissociating Folate Receptors - B-sites

A low density of slow-dissociating folate receptors was detected on the surface of vegetative amoebae by using the B-site assay of De Wit and Van Haastert (1985). For this assay, cells were incubated with [ $^3$ H]folate as previously described (chapter 3), then after 120sec incubation the cells were diluted with a solution of excess (25 $\mu$ M) unlabelled folate to dissociate [ $^3$ H]folate from all fast-dissociating forms of the receptor. After 60sec in the dissociation solution, cells with residual bound [ $^3$ H]folate attached to slow-dissociating surface receptors (B-sites) were recovered by centrifugation through silicone oil. All stages of the assay were performed at 4 $^{\circ}$ C. Assay details are described in chapter 2.

Figure 7.1 shows that [ $^3$ H]folate remains bound after 60sec dissociation with unlabelled folate (25 $\mu$ M). Tight binding of folate was sensitive to competitive inhibition by isotopic dilution with unlabelled folate. This was demonstrated by including various concentrations of unlabelled folate in the initial incubation solution; the unlabelled folate competed for binding to the B-sites as well as binding to all

Table 7.1

DISSOCIATION CONSTANTS OF FOLATE RECEPTORS\*  
(De Wit, et al., 1985)

Ligands	A-sites ( $A^L, A^H$ )	B-sites	C-sites ( $C^F, C^S$ )
FA	70-450	17	15-50
$N^{10}$ -CH <sub>3</sub> -FA	700-4500	4	2.8-20
DAFA	55-370	240	6300-11,000
Methotrexate	70-450	20,000	6000-25,000
Aminopterin	40-300	>30,000	>30,000
Pterin	>30,000	>30,000	>30,000
p-ABA-glu	>30,000	>30,000	>30,000

\* All  $K_d$  values are in nmoles per litre.

7    HIGH AFFINITY, SLOW-DISSOCIATING FOLATE RECEPTORS —  
      THE CHEMOTACTIC RECEPTOR

7.1    Introduction

As this study was nearing completion, other researchers reported very complex binding characteristics for the folate receptor (De Wit and Van Haastert, 1985; De Wit et al., 1985). They described five classes of receptor sites that bind folates with variable affinities or variable dissociation kinetics (table 7.1). The predominant class of receptors ( $150,000 \text{ sites cell}^{-1}$ ), designated A-sites by De Wit and Van Haastert (1985), possess characteristics that are identical to the folate receptors that I have described throughout this study. The receptor class of particular interest in the report, termed B-sites, were a group present at 550 sites per cell. By correlating the chemotaxis-inducing activity of certain folate analogues with their binding to B-sites, De Wit suggested that these high-affinity receptors were the functional receptors responsible for initiating chemoattractant signal transduction. A third class of receptors, called C-sites, were detected in the presence of high DAFA concentrations ( $3.3\mu\text{M}$ ) and were present at a density of  $1500 \text{ sites cell}^{-1}$ . The remaining two classes described were an altered-affinity sub-class of the A-sites ( $A^H$ ) and a slow-dissociating form of the C-sites ( $C^S$ ). To add yet another level of complexity, these workers have suggested that receptor classes may not exist as fixed-affinity discrete groups of receptors; alternatively, they propose that the receptor classes arise from a homogeneous pool of receptors (perhaps the A-sites) and are detected only after ligand-induced receptor modulation.

In this chapter, I have employed the B-site assay (De Wit et al., 1985) to answer several questions: a) Do B-sites exist on D. discoideum, strain NC-4? b) Do any of the receptor sites detected show

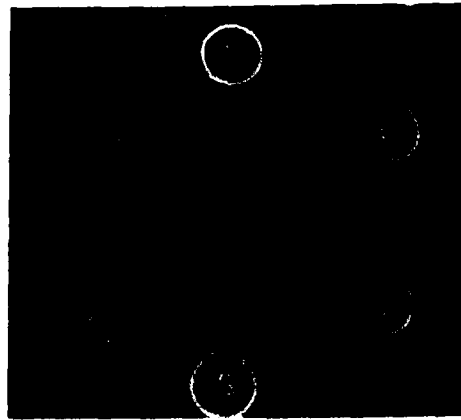
## *Chapter 7*

detection of diffusing chemoattractant and the area of most rapid cell migration. As cells migrate away from the central spot, Grutsch et al. (1985) postulate that the migration rate will gradually slow down in response to a slower rate of enzyme degradation of the chemoattractant. Consequently, the dense leading edge of migrating cells will develop and become more dense as new cells speed from the central spot. If this were true, then reducing the concentration of chemoattractant in the agar would allow an increase in the rate of chemoattractant breakdown since the enzyme to substrate ratio would become more favorable and one should observe a less dense front of migrating cells. Such a trend is seen for the L-monapterin dose response series where a much less dense band of leading cells develops with  $10^{-7}$  and  $10^{-8}$  M L-monapterin than with higher concentrations (fig 6.5).

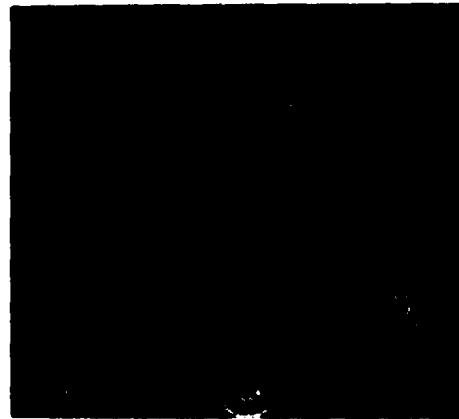
A possible explanation of the L-monapterin chemotactic response is that the 12hr actin-accumulation response is mediated by a pterin binding receptor. The fact that folate induces actin accumulation in the cytoskeleton at 12hr could be explained if the pterin receptor also bound folate, or if the folate used contained pterins in the form of breakdown products or contaminants. This L-monapterin response is studied in more detail in the next two chapters.

[L-Monapterin]

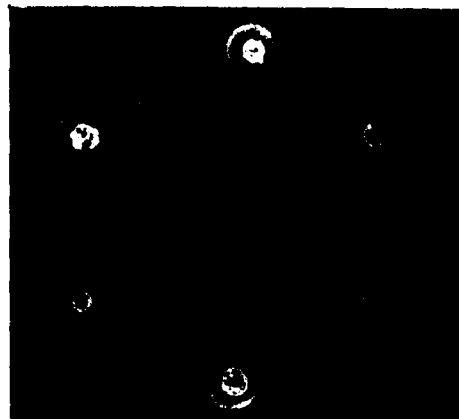
$10^{-5} \text{ M}$



$10^{-6} \text{ M}$



$10^{-7} \text{ M}$



$10^{-8} \text{ M}$



Fig 6.5

Figure 6.5    Effect of L-monapterin Dose on the  
Chemotactic Response of Developing 12hr Cells

Vegetative amoebae were developed for 12hr in shaker culture then spotted onto agar containing L-monapterin ( $10^{-8}$ - $10^{-5}$ M) and allowed to migrate for 24hr. Photographs are representative examples from three separate experiments.

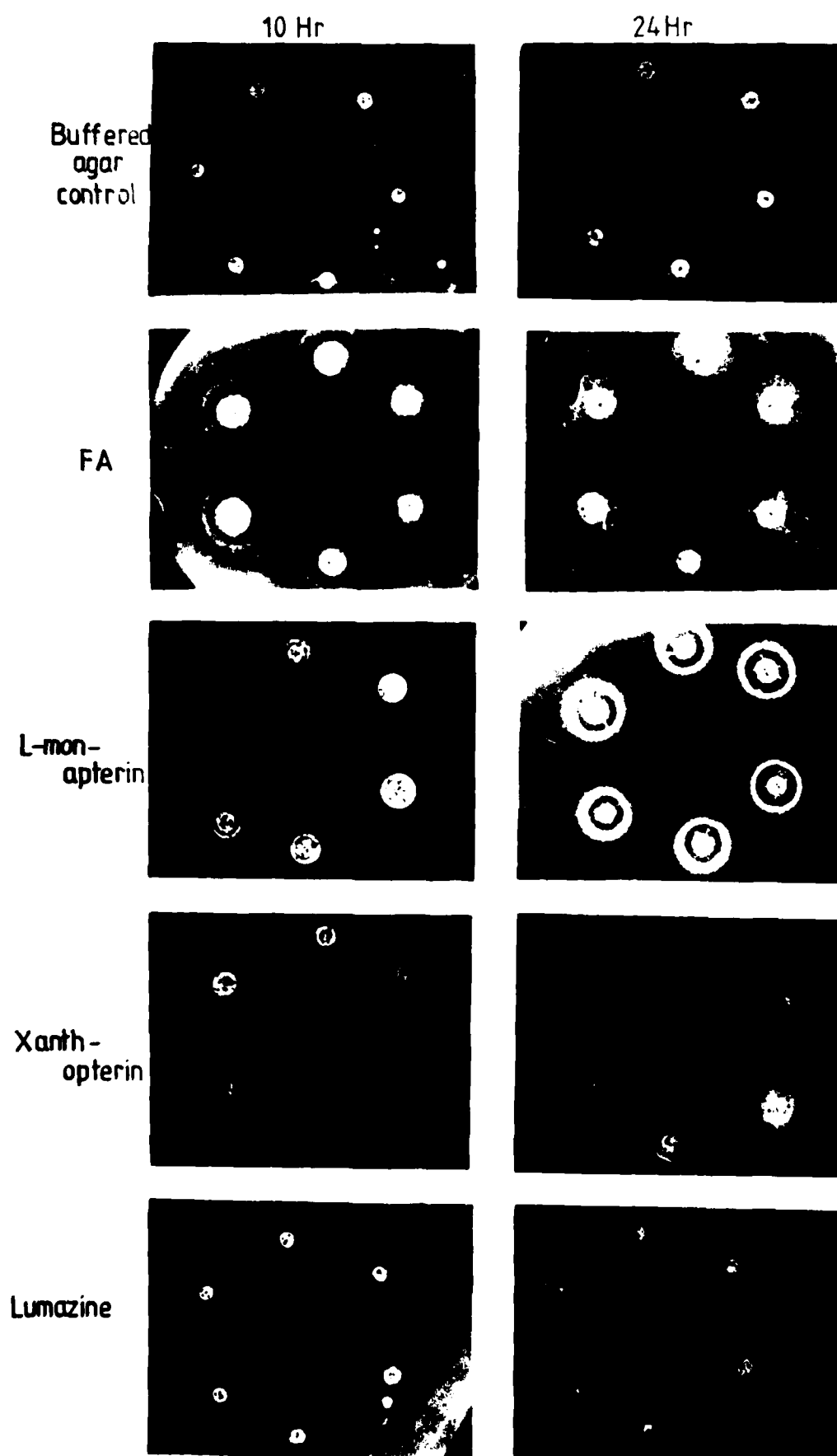


Fig. 6.4

Figure 6.4     Chemotactic Response to L-monapterin by  
Developing 12hr Cells

Vegetative amoebae were spotted onto agar containing folate, L-monapterin, xanthopterin, or lumazine (all chemoattractants at 10uM) and allowed to migrate for 24hr. Results are representative examples from three separate experiments.

detect the true chemotactic receptor or, there are interconvertible classes of receptors and the folate receptor detected by my assay is modulated to an altered receptor form (perhaps with altered affinity and dissociation characteristics) after ligand binding. If the second alternative is true, then one could speculate that only certain ligands allow the receptor to attain this modulated form and only these ligands then elicit a chemotactic response. This intriguing prospect will be discussed in more detail later in this thesis.

#### D Chemotactic Response of Developing 12hr Cells Toward Folate and L-monapterin

A surprising result was observed while testing vegetative cells against pterin analogues by the "spreading" assay. The modified pterin, L-monapterin, showed no chemotactic activity with vegetative cells (figure 6.1). But, interestingly, if these cells were allowed to develop for 24hr on buffered agar containing this chemoattractant, the cells acquired chemotactic responsiveness at about 12hr of development. This response was quite evident if the cells were allowed to continue development and migration through 24hr (figure 6.4). The chemotactic response to L-monapterin after 12hr of development was dose dependent with optimum sensitivity at  $10^{-6}$ - $10^{-7}$ M (figure 6.5).

Grutsch et al. (1985) have provided an explanation for the dense leading edge of chemoattractant-induced migrating cells as seen for L-monapterin (figure 6.4 and 6.5). They have proposed that cells will only move in response to a chemoattractant gradient and a chemoattractant gradient will be formed only if a sufficiently high enzyme concentration exists to degrade the available chemoattractant. In the "spreading" spot assay, the highest enzyme concentration will be adjacent to the central dense cell spot and will then provide the steepest gradient of chemoattractant, the area for most sensitive

or 12hr of development. However, attempts to reproduce the result which suggested that B-sites were retained during development failed in three additional experiments. In these follow-up experiments, all [ $^3\text{H}$ ]folate binding was sensitive to dissociation by 25uM folate suggesting the complete absence of B-sites after the vegetative stage (data not shown).

### 7.3 Discussion

De Wit's proposed folate receptor family resulting from receptor modulation after ligand-receptor association may explain the inconsistencies observed between folate receptor binding (A-sites) and chemotactic responses in D. discoideum (Pan and Wurster, 1978; Wurster and Butz, 1980; Van Haastert et al., 1982c; chapter 3; chapter 6). The A-site binding data presented by De Wit et al. (1985) shows ligand receptor specificities nearly identical to those presented in chapter 5 (cf. table 7.2 and figure 5.6). However, as mentioned previously, these specifications do not correspond with vegetative cell chemotaxis activities toward these ligands. DAFA binds with high affinity (equivalent to folate) yet does not induce chemotaxis;  $\text{N}^{10}\text{-CH}_3\text{-folate}$  binds with reduced affinity relative to folate yet is a strong chemoattractant; methotrexate and aminopterin bind with high affinity yet elicit abnormal chemotactic responses (chapter 6, fig. 6.2). The reported B-sites show altered affinities for several ligands when compared to A-site binding (table 7.1). The B-sites were shown to bind DAFA, aminopterin and methotrexate with a much lower affinity than folate and bound  $\text{N}^{10}\text{-CH}_3\text{-folate}$  with an affinity even higher than that for folate. These binding characteristics fit quite well with the proposed functional role for these B-sites in chemoattractant signal transduction. The C-sites showed binding affinities for folate derivatives that were nearly identical to those for B-sites and, consequently, could also function in chemotaxis.

In leucocytes multiple receptor classes have also been reported and it was further proposed that these receptor classes might transduce specific, independent cell responses (Snyderman et al., 1984). Such functional roles in D. discoideum for these separate classes of folate receptors are an interesting prospect. If the A-sites do not function in chemotaxis, then they may play a role in other transduction-related events. De Wit et al. (1985) have suggested that the A-sites might function in inducing cAMP synthesis and secretion (Devreotes, 1983) since methotrexate strongly inhibits folate induced cAMP secretion. As previously proposed from the chemotaxis data (chapter 6), the A-sites may function in chemokinesis, by transduction of a signal which directs an increase in migration rate without cell orientation. The high binding affinities for methotrexate and aminopterin to my folate receptors (A-sites?) and the chemokinetic-like response by vegetative amoebae toward methotrexate and aminopterin allows me to propose such a role for the A-sites. In time, other ligand-initiated cell responses such as cGMP production, phosphodiesterase induction and contact sites-A development may perhaps be correlated with a specific folate receptor class.

De Wit and Van Haastert (1985) reported that receptor modulation occurred in the high density A-site class. Experiments measuring binding kinetics allowed these workers to propose a half-time of 7-10sec for conversion of the low-affinity A-sites ( $A^L$ ,  $K_d=4-500nM$ ) to the high-affinity A-sites ( $A^H$ ,  $K_d=50-90nM$ ). Additionally, De Wit proposed that the B-sites and C-sites may arise from another receptor class (possibly the A-sites). It is tempting to hypothesize how such ligand induced increases in affinity might occur. De Wit has proposed two models: 1) Positive cooperativity, where an allosteric-like site, if occupied, will alter the affinity of the binding site; 2) Conformational changes, such that after occupation with ligand, the binding protein assumes an

energetically more favourable conformation which then shows a higher ligand-binding affinity. Although these two models may not necessarily be mutually exclusive, ligand induced affinity changes seem common to many receptor systems, including  $\beta$ -adrenergic receptors (Toews et al., 1983), acetylcholine receptors (Boyd and Cohen, 1980), N-formyl peptide receptors on granulocytes (Jesaitis et al., 1984) and the cAMP receptor in D. discoideum (Van Haastert, 1984). Such affinity modulations may result from receptor interactions with other membrane proteins, such as a GTP-binding protein (Molinoff et al., 1981; De Wit and Bulgakov, 1985; Van Haastert, 1984), or as introduced previously (chapter 4) by association of the receptor with cytoskeleton-associated effector molecules or with the cytoskeleton directly (Jacobs and Cuatrecasas, 1976; Schechter and Bothwell, 1981; Jesaitis et al., 1984). The ligand-induced actin-accumulation response in D. discoideum first described by McRobbie and Newell (1983) and studied in further detail in the next chapter, may be a manifestation of such a receptor-cytoskeleton interaction.

## *Chapter 8*

## 8 THE ACTIN RESPONSE -- ACTIN ACCUMULATION IN THE CYTOSKELETON AFTER FOLATE STIMULATION

### 8.1 Introduction

The term cytoskeleton describes the collection of filamentous proteins which determine the shape and structure of the cell and is operationally defined as the insoluble residue that remains after non-ionic detergent extraction (Fulton, 1984). The components of the D. discoideum cytoskeleton include actin containing microfilaments with associated myosin (Wooley, 1972; Spudich, 1974), tubulin-containing microtubules (Cappuccinelli et al., 1978; White et al., 1983), possibly intermediate filaments (Koury and Eckert, 1983) and many associated binding proteins (reviewed by Spudich and Spudich, 1982). As previously shown in platelets (Carlsson et al., 1979; Markey et al., 1981), in leukocytes (White et al., 1982; Rao and Varani, 1982) and in D. discoideum (McRobbie and Newell, 1983, 1984a), upon stimulation with the cell specific chemoattractant, there is a rapid redistribution of detergent-soluble G-actin into the detergent-insoluble F-actin-containing cytoskeleton. Such changes in actin distribution may be related to cell locomotion (Howard and Meyer, 1984). In a related study Condeelis (1979) demonstrated possible interactions of concanavalin A (ConA) receptors with the actin cortical matrix. These results may reflect receptor-cytoskeleton interactions that are in some way involved in signal transduction (Spudich and Spudich, 1982).

The actin response seen in D. discoideum is manifested as an increase in insoluble actin associated with the isolated cytoskeleton after ligand stimulation (McRobbie and Newell, 1983, 1984a). Prior to the discovery of the actin response in slime moulds, the only other well-defined cellular responses available for study were chemotaxis, light scattering responses, cGMP accumulation and cAMP secretion

(relay). The actin response provided yet another avenue of research to unravel the mechanism of signal transduction. By studying the actin response, McRobbie and Newell (1983) discovered that folate elicited a response at 12hr of development. I have, therefore, used the actin assay to further characterize this folate-induced response during cellular slime mould development.

## 8.2 Results

### A Folate-Stimulated Actin Response in Developing Cells Isolated from a Solid Substratum

The early results of ligand-induced actin responses were obtained from cells that were allowed to develop during starvation in shaken cell suspensions (McRobbie and Newell, 1983). To verify the 12hr folate-induced response, I allowed D. discoideum amoebae, strain NC4, to develop on a solid substratum (Millipore filters) and then stimulated the cells with folate (50uM) at stages of development from 0-12hr. The results in figure 8.1 show that there was a folate-induced actin response with 0hr cells; the cells became unresponsive to folate during the aggregation phase (8hr); and then, as previously reported, a renewed actin response was detected at 10-12hr which again subsided by 14hr. The control response of 12hr cells to SORPHOS stimulation is compared with the 12hr folate-induced response in figure 8.1. The 0hr response was somewhat different from that previously reported by McRobbie and Newell (1983). The early 3-5sec response was not well defined but the characteristic "trough" seen at 10-15sec was always detected. This trough has been correlated with the "cringe" response which is seen as a rapid contraction and rounding of the cells after chemoattractant stimulation (Futrelle et al., 1982; McRobbie, 1984).

At the 12hr stage of development the aggregates had just begun to develop tips; it was at this stage that the folate-elicited actin

Figure 8.1     Developmental Regulation of the Folate-  
elicited Actin Response

D. discoideum cells developing on a solid substratum were harvested at various times (0-14hr), suspended at  $1 \times 10^8$  cells  $\text{ml}^{-1}$  and then stimulated with folate ( $50 \mu\text{M}$ ). Triton-insoluble cytoskeletons were prepared at preset times (0-30sec) after folate stimulation. Actin content was determined by one dimensional SDS-PAGE and scanning densitometry. Content is expressed as percent change in actin from the 0sec basal value. Data for each time period are means from at least three separate experiments. Control response with SORPHOS at 12hr is shown by the open circles.

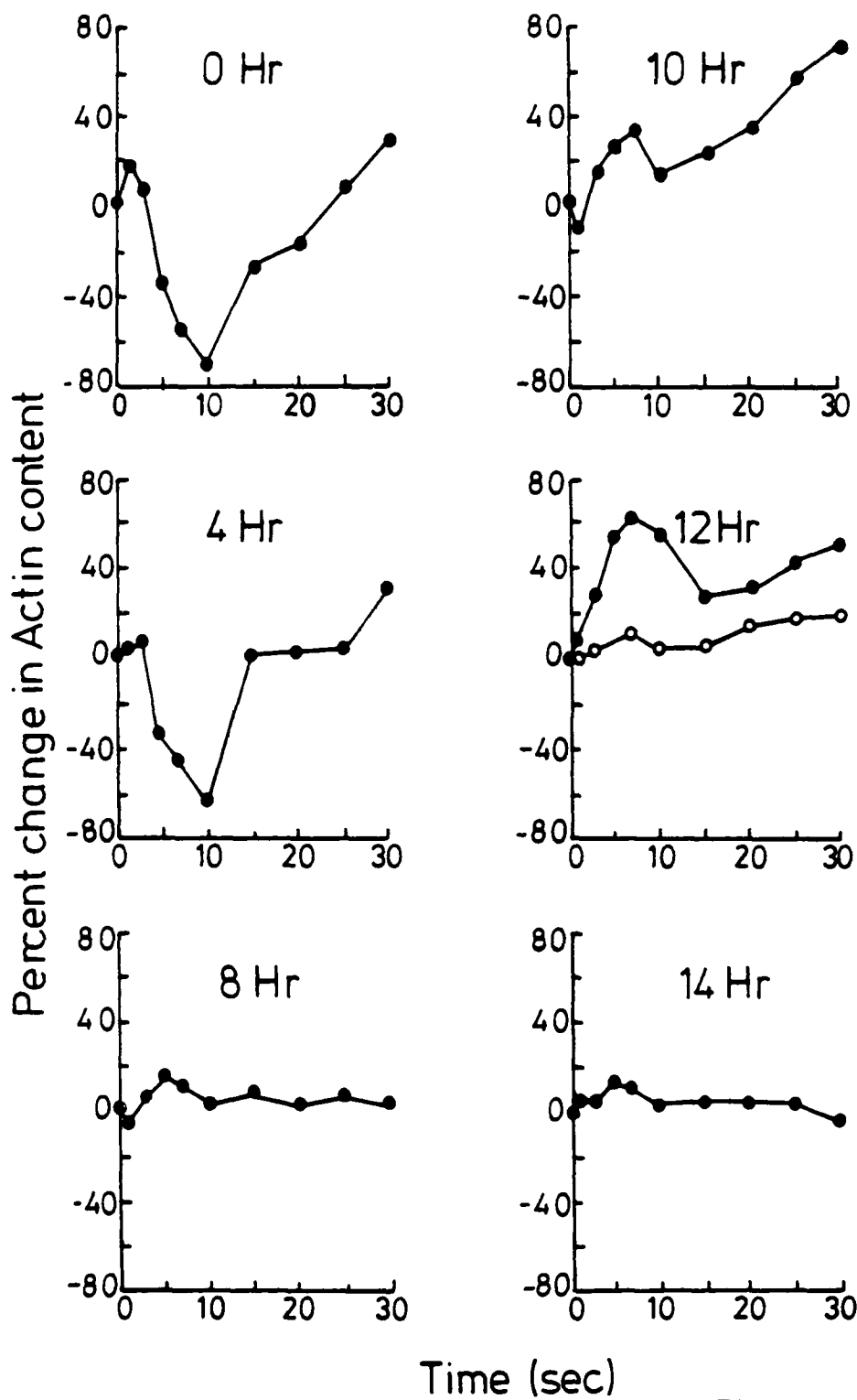


Fig. 8.1

response was strongest. Prior to tip formation (10hr) the elicited response was much weaker and by 14hr, when the tipped aggregates had elongated into standing fingers, the actin response was no longer detected. The differences in the amount of actin extracted from 12hr tipped aggregates and 14hr standing fingers can be seen in figure 8.2.

#### B Ligand Specificity of the 12hr Actin Response

The chemotaxis results of chapter 6 prompted me to evaluate the specificity of the 12hr actin response by stimulating developing cells (12hr) with a variety of chemoattractants. Development of cells used in this experiment and subsequent experiments was induced by starvation in shaker culture rather than on filters since the cells from shaker development were easier to employ routinely and showed the same developmental timing of the folate/actin response as did cells from a solid substratum. Cells developed for 12hr in shaker culture, known to be chemotactically responsive to folate and L-monapterin but not responsive to lumazine, xanthopterin or DAFA (chapter 6), showed a corresponding pattern of sensitivities in the actin response assay (figure 8.3). Only folate and L-monapterin (each at 50 $\mu$ M) induced increased levels of actin in the cytoskeletons of 12hr cells.

#### C The L-monapterin-Elicited Actin Response is Developmentally Regulated

Chemotaxis studies had also revealed that vegetative D. discoideum cells were not chemotactically responsive to L-monapterin but developed responsiveness after 12hr of development (chapter 6). To determine if the actin response was also developmentally regulated, D. discoideum cells at different stages of development were stimulated with L-monapterin (10 $\mu$ M) and the cytoskeleton-actin content measured. As predicted from the chemotaxis data, vegetative and aggregating (4-8hr) cells were unresponsive to L-monapterin stimulations but cells became responsive at 12hr of development (figure 8.4).

Figure 8.2     The Actin Response at  
12hr and 14hr of Development

- a) Coomassie stained SDS-polyacrylamide gel (10%) of Triton-insoluble cytoskeletons prepared at preset times following folate (50 $\mu$ M) stimulation of cells from 12hr aggregates (lanes 2-5) and 14hr aggregates (lanes 6-9) from a solid substratum.

Lane 1- molecular weight standards

Lanes 2-5 - cytoskeletons from 12hr cells

- 2 - 0 sec (prior to stimulation)
- 3 - 1 sec after stimulation
- 4 - 7 sec after stimulation
- 5 - 10 sec after stimulation

Lanes 6-9 - cytoskeletons from 14hr cells

- 6 - 0 sec (prior to stimulation)
- 7 - 1 sec after stimulation
- 8 - 7 sec after stimulation
- 9 - 10 sec after stimulation

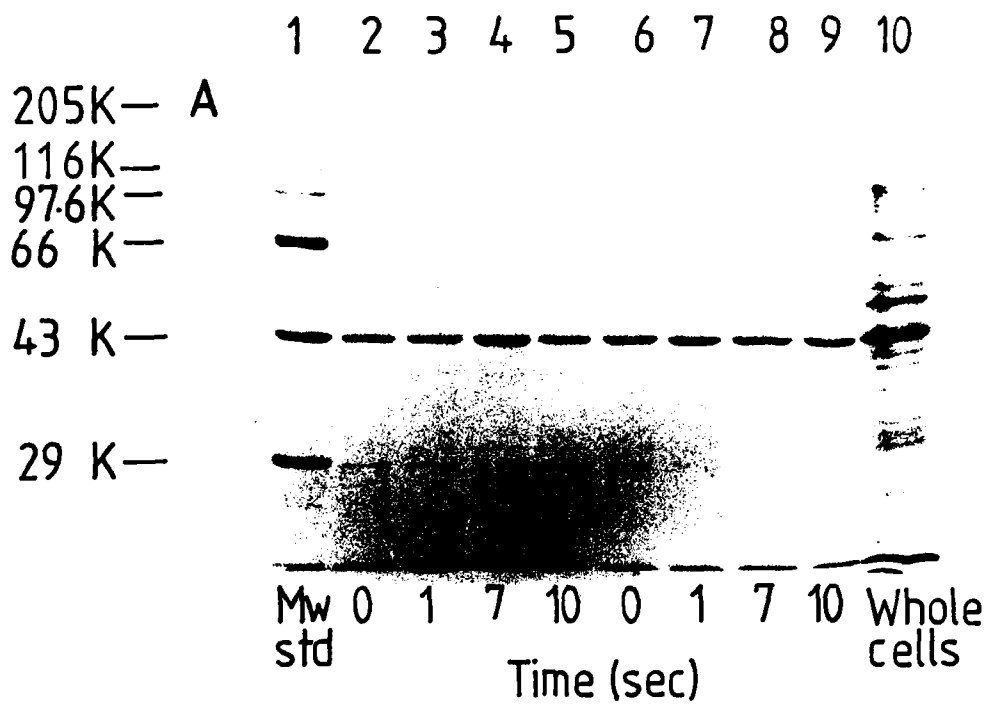
Lane 10 - whole cell lysate

- b) SDS-polyacrylamide gel (10%) of Triton-insoluble cytoskeletons prepared at 0-30sec after folate stimulation (50 $\mu$ M). Results from scanning densitometry analysis of this representative gel appear in figure 10.1c.

Cytoskeleton extracts =  $4 \times 10^6$  cytoskeletons/lane

Whole Cell Lysate -  $2 \times 10^6$  cells/lane

Molecular weight standards are described in chapter 2.



B

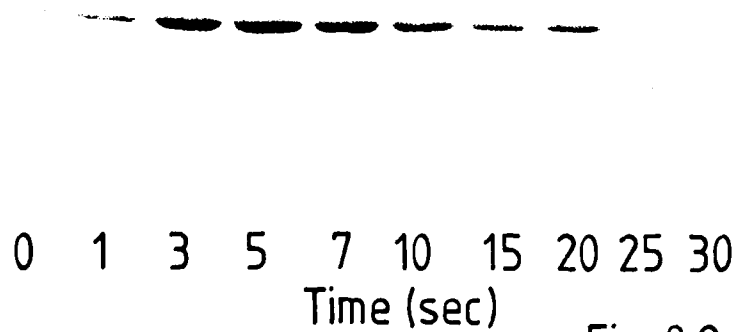


Fig 8.2

Figure 8.3     Ligand Specificity of the  
12hr Actin Response

D. discoideum cells (12hr, shaker starvation) were washed and resuspended at  $1 \times 10^8$  cells/ml, stimulated with various ligands ( $10 \mu\text{M}$ ), and then Triton-insoluble cytoskeletons were prepared at preset times (0-30sec) following ligand stimulation. Actin content was measured by PAGE and scanning densitometry. Actin content is expressed as percent change from 0sec basal values.

- a) Folate-elicited actin response
- b) L-monapterin-elicited actin response.
- c) Lumazine-elicited actin response.
- d) Xanthopterin-elicited actin response.

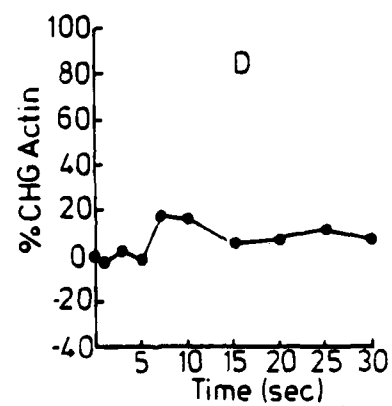
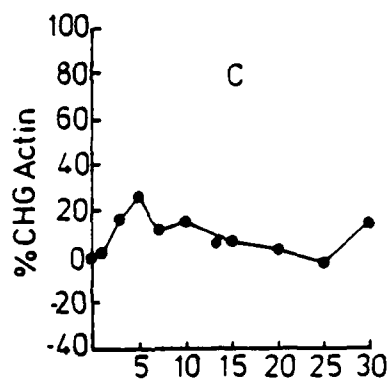
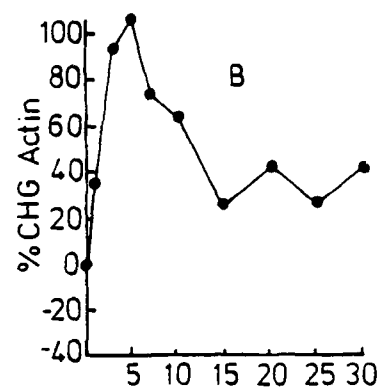
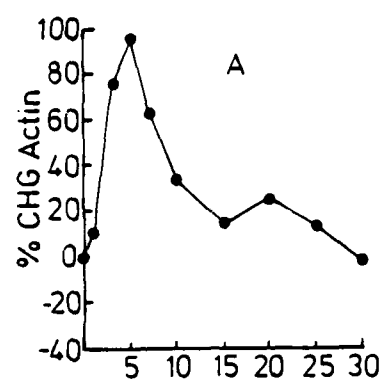


Fig. 8.3

Figure 8.4     Time Course of L-monapterin-elicited  
Actin Response

D. discoideum cells developed by starvation in shaker culture (0-12hr) were harvested, resuspended at  $1 \times 10^8$  cells  $\text{ml}^{-1}$ , and stimulated with L-monapterin ( $10 \mu\text{M}$ ).

Each graph is a representative example of two separate experiments.

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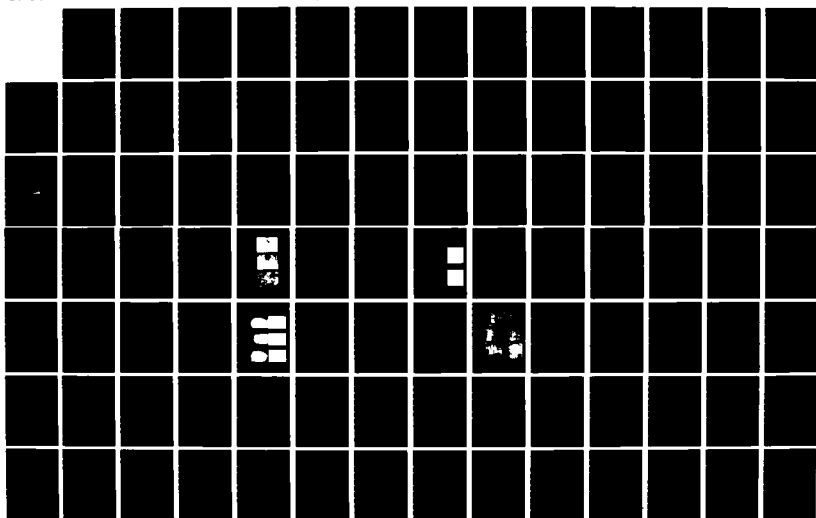
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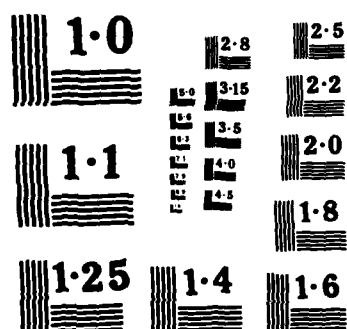
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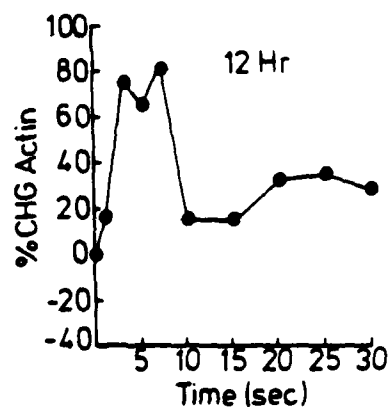
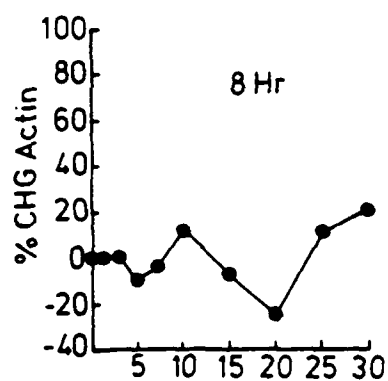
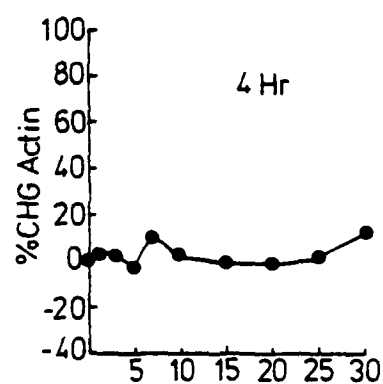
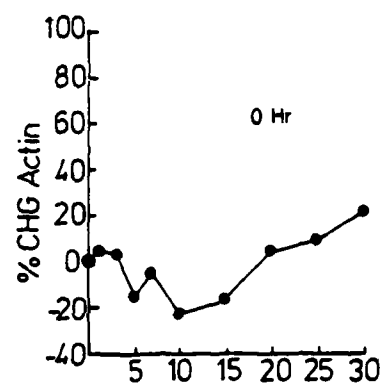


Fig. 8 4

#### D The Effect of L-monapterin Dose on the Actin Response

Low concentrations of L-monapterin ( $10^{-8}$  M) still induced a dramatic increase in cytoskeletal-actin accumulation (figure 8.5a) as predicted from chemotaxis data (figure 6.4b). In comparison, folate concentrations below  $10^{-7}$  M failed to induce an actin response (figure 8.5b) and responses in general were quantitatively lower than those with L-monapterin. Only a 60% actin increase was seen with  $10^{-5}$  M folate whereas  $10^{-5}$  M L-monapterin induced a 120% increase in cytoskeletal-actin.

#### E L-monapterin-Induced Actin Response with a Folate Antagonist Present

The differential effects of folate and L-monapterin dose on the actin response suggested that the L-monapterin-induced response was possibly mediated by a separate pterin receptor or that L-monapterin was the authentic ligand for the 12hr response and the weaker folate response was simply a cross-reactive response. To test these possibilities, I stimulated 12hr cells with  $10^{-5}$  M L-monapterin against a constant background of  $10^{-4}$  and  $10^{-5}$  M folate (figure 8.6). For controls, the actin response to folate ( $10^{-5}$  M) was observed against a constant background of either  $10^{-4}$  M folate or  $10^{-4}$  M L-monapterin (figure 8.6). These results suggest that the L-monapterin- and folate-induced responses are mediated by separate receptors since either response was still evident even though the alternative antagonist was present in high concentration. Additionally, these results imply that the putative L-monapterin receptor is more sensitive to folate inhibition than the folate receptor is to L-monapterin inhibition.

### 8.3 Discussion

The folate-induced actin response correlated with tipped-aggregate

Figure 8.5      Effect of Folate and L-monapterin Dose  
on the Actin Response

D. discoideum cells developed for 12hr by starvation in shaker culture were suspended at  $1 \times 10^8$  cells  $\text{ml}^{-1}$  and stimulated with various concentrations of ligand ( $10^{-8}$ - $10^{-5}$  M). Triton-insoluble cytoskeletons were prepared at preset times (0-30sec) after stimulation and the actin content determined by PAGE and scanning densitometry.

- a) Effect of folate dose on actin response
- b) Effect of L-monapterin dose on actin response

Each graph is a representative example of two separate experiments.

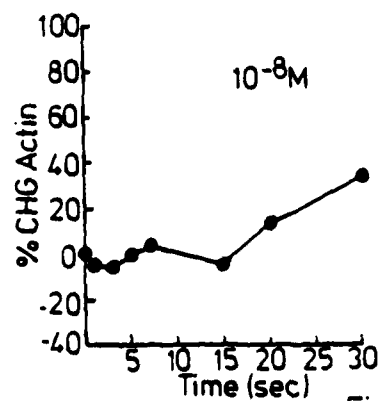
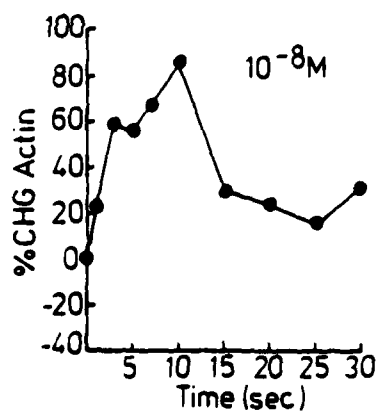
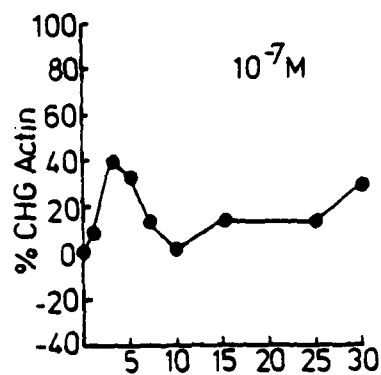
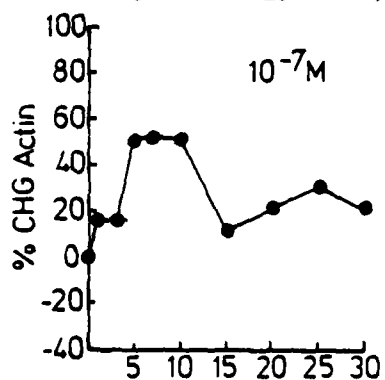
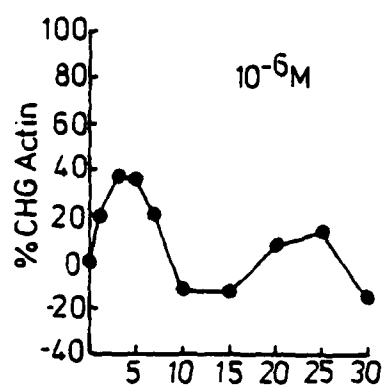
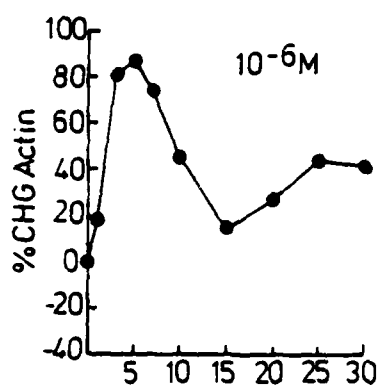
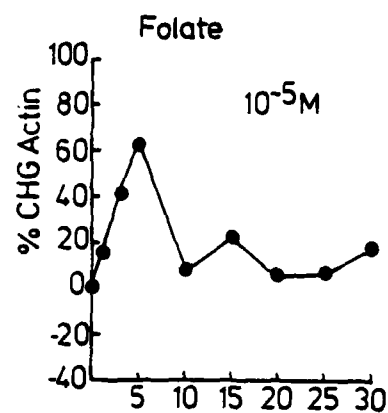
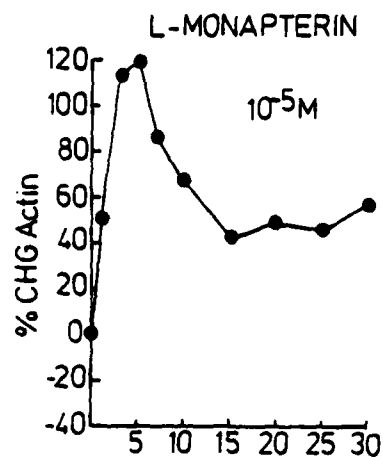


Fig 8.5a

Fig.8.5b

Figure 8.6    Effect of Antagonists on the Folate- and  
L-monapterin-elicited Actin Responses

D. discoideum cells, developed 12hr by starvation in shaker suspensions and bathed in a background of antagonist ( $10^{-5}$ - $10^{-4}$ M), were stimulated with ligand ( $10^{-5}$ M) and then Triton-insoluble cytoskeletons were prepared at preset times (0-30sec) after stimulation.

- a) Ligand =  $10^{-5}$ M L-monapterin  
Antagonist background =  $10^{-4}$ M folate
- b) Ligand =  $10^{-5}$ M L-monapterin  
Antagonist background =  $10^{-5}$ M folate
- c) Ligand =  $10^{-5}$ M folate;  
Antagonist background =  $10^{-4}$ M folate
- d) Ligand =  $10^{-5}$ M folate  
Antagonist background =  $10^{-4}$ M L-monapterin

Each graph is a representative example of two separate experiments.

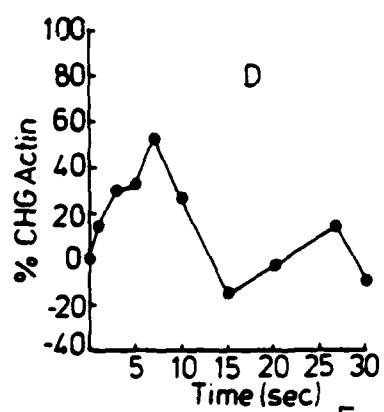
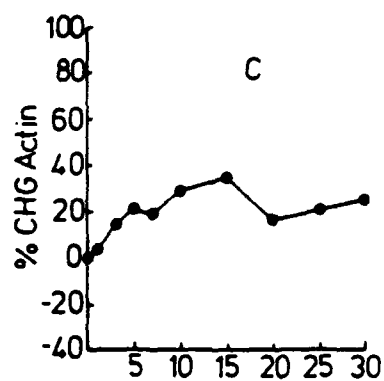
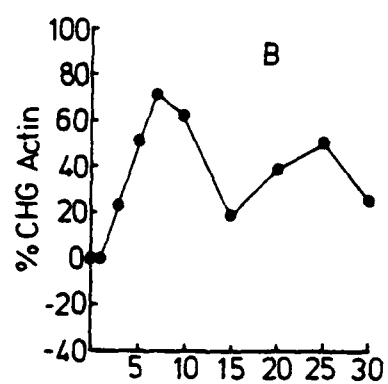
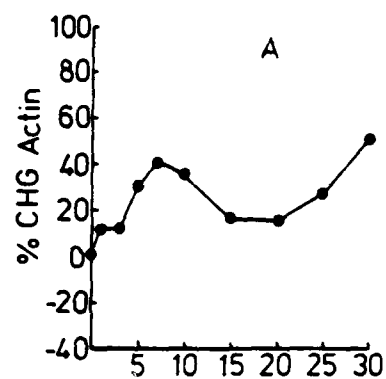


Fig 8.6

formation when cells were developed on a solid substratum and the specificity of this response correlated with the chemotaxis data. Folate and L-monapterin, known to induce chemotaxis of 12hr cells, also induced an actin response at this stage of development (figure 8.3). Lumazine, xanthopterin and DAFA, stereochemical analogues of L-monapterin and folate, elicited very weak actin responses as predicted from the previous chemotaxis data (figure 6.4a).

The L-monapterin-induced actin response showed developmental regulation with a strong response detected at 12hr of development (figure 8.4). The actin response was elicited with quite low concentrations of L-monapterin ( $10^{-8}$  M) which again supported previous chemotaxis dose response data. L-monapterin-induced actin response studies, conducted in a background of folate as an antagonist, suggested that the folate and L-monapterin responses may be mediated by separate receptors. The putative L-monapterin receptor displayed some sensitivity to folate inhibition but the folate receptor appeared to be insensitive to L-monapterin as an antagonist.

It was originally hypothesized (chapter 1) that tip formation was the result of a signal transduction event (communication event). As discussed in chapter 1, two criteria had to be met before this hypothesis could be proven. First, receptors capable of detecting the chemical message had to be present on the surface of the receiving cells. Secondly, these cells had to demonstrate chemoattractant mediated responses.

The presence of folate receptors was studied in previous chapters (3,4,7). Although no additional folate receptors of either class, A-sites (chapter 4) or B-sites (chapter 7), reappeared at 12hr of development, the residual receptors (A-sites) retained during development may be sufficient to mediate folate responses related to tip formation. Additional support for the presence of folate receptors can

be drawn from my studies on the specificity of the actin response. The actin response appears quite sensitive to minor alterations in the chemical structure of the stimulants, and the results from the antagonist experiments show that the folate-elicited actin response is not sensitive to L-monapterin inhibition even though L-monapterin elicits an even stronger response than folate (figure 8.5). Both of these results provide further evidence for folate receptor involvement in the 12hr actin response and possibly in tip formation. Therefore, the first aim of this study, demonstrating the involvement of folate receptors in tipped aggregate development, has provisionally been met.

Two chemoattractant mediated responses have been observed in 12hr developing aggregates, thereby suggesting that tip formation involves a signal transduction event. Twelve hour cells demonstrate a chemotactic response to folate and L-monapterin and these two ligands also elicit the accumulation of additional actin in the cytoskeleton. It cannot be certain that the actin response is indicative of a cell movement event, but based on the assumption that the cytoskeleton is the motile apparatus one could propose that changes in the cytoskeleton configuration or composition are related to cell movements. The temporal correlation of tipped aggregate formation at 12hr of development with the reappearance of the actin response at this stage suggests that tip formation involves a cytoskeleton-related event. The fact that the ligand specificity of the actin response correlates with ligand specificity of chemotaxis suggests that cell movement and the actin response are related. The similar dose responses to L-monapterin in both chemotaxis and the actin response are also indicative that tip formation involves a chemotactic event.

To further establish that the actin response was evidence for a cell movement event, it became necessary to look for other molecular

responses thought to implicate signal transduction from cell surface receptors to the intracellular components controlling cell movement. cGMP accumulation occurs after chemoattractant stimulation (Wurster et al., 1977; Mato et al., 1977) and internal  $\text{Ca}^{2+}$  mobilization may mediate the cGMP response (Europe-Finner and Newell, 1984). The relation of these responses to tip formation in the 12hr aggregate are discussed in the two chapters to follow.

## *Chapter 9*

9 THE FOLATE-STIMULATED cGMP RESPONSE IN DICTYOSTELIUM DISCOIDEUM  
DURING DEVELOPMENT

9.1 Introduction

Rapid and transient increases in intracellular cGMP levels occur after stimulation of cellular slime mould cells with species-specific and developmental stage-specific chemoattractants. Vegetative D. discoideum cells accumulate peak levels of cGMP within 10sec after folate stimulation and aggregation competent D. discoideum cells respond in a similar manner to cAMP stimulations (Mato et al., 1977; Wurster et al., 1977). The resting cGMP level in D. discoideum is approximately 1 pmole cGMP per  $10^7$  cells and this level increases to 6-10 pmole following stimulation. Similar cGMP responses have been found in other slime mould genera and species: e.g. Polysphondylium violaceum stimulated with glorin (Wurster et al., 1978), D. lacteum stimulated with L-monapterin (Mato and Konijn, 1977b), and recently D. minutum (16hr stage of culmination) stimulated with cAMP (Schaap and Wang, 1985). A class of "streamer" mutants have been described which appear altered in their chemotactic (movement) and cGMP responses (Ross and Newell, 1981) as a result of aberrant cGMP phosphodiesterase activity (Van Haastert et al., 1982a; Coukell et al., 1984). This data from the studies on the "streamer" mutants and the general occurrence of the cGMP response suggest that the temporally controlled cGMP pulses may be a sequential event in signal transduction leading to chemotaxis during cell aggregation and perhaps chemotaxis during slime mould morphogenesis (Schaap and Wang, 1985).

To determine if the actin response was indicative of signal transduction and chemotaxis during tip formation, I studied the folate-induced cGMP response in developing cells.

## 9.2 Results

### A Folate-Stimulated cGMP Responses During Development of Wild-type D. discoideum Cells

Vegetative D. discoideum amoebae of strain NC4 were harvested from clearing mass plates and either used immediately for 0hr studies or allowed to starve on a solid substratum (Millipore filters) until they reached the desired stage of development. Vegetative cells were pre-stimulated with folate to induce cell synchronization prior to use in the cGMP assay (chapter 2). Since folate stimulations have been shown to delay the developmental loss of folate responses (Van Haastert, 1983), the cGMP response in developing cells was measured both with and without folate pre-stimulation. Both methods produced the same result.

For the cGMP assay, synchronized vegetative cells or developing cells were stimulated with folate (50 $\mu$ M), lysed at pre-set times (0-3lsec) with perchloric acid (1.6% v/v) and then cell-free supernatants were analyzed for cGMP content. cGMP levels were measured with a radioimmunoassay (Amersham) modified as previously described (Van Haastert et al., 1981, chapter 2).

Figure 9.1 shows cGMP responses for cells from 0-14hr of development. In 0hr NC4 cells, cGMP increased from a basal level of 0.5pmoles per 10<sup>7</sup> cells to 4.5pmoles at 12sec after stimulation. Cells after 8hr of development demonstrated a cGMP response equivalent to 30-40% of the 0hr response. Ten-hour cells showed no increase in cGMP levels after folate stimulation and a renewed response was not detected through 14hr of development.

Figure 10.2    TMB-8 Inhibition of the Actin Response

After 12hr of development in shaker culture, cells of strain NC4 were suspended at  $10^8$  cells  $\text{ml}^{-1}$  and incubated for an additional 40min in either SORPHOS or with 1mM TMB-8 in SORPHOS. Following TMB-8 or buffer treatment, Triton-insoluble cytoskeletons were prepared at preset times following folate ( $50\mu\text{M}$ ) stimulation.

Cytoskeleton samples were separated on an SDS-polyacrylamide gel (10%) and the gel was stained with Coomassie blue.

Lanes 1-4 - SORPHOS control samples

Lane 5 - Molecular weight standards

Lanes 6-9 - 1mM TMB-8 treated samples

Cytoskeleton extracts =  $4 \times 10^6$  cytoskeletons/lane

Molecular weight standards are described in chapter 2.

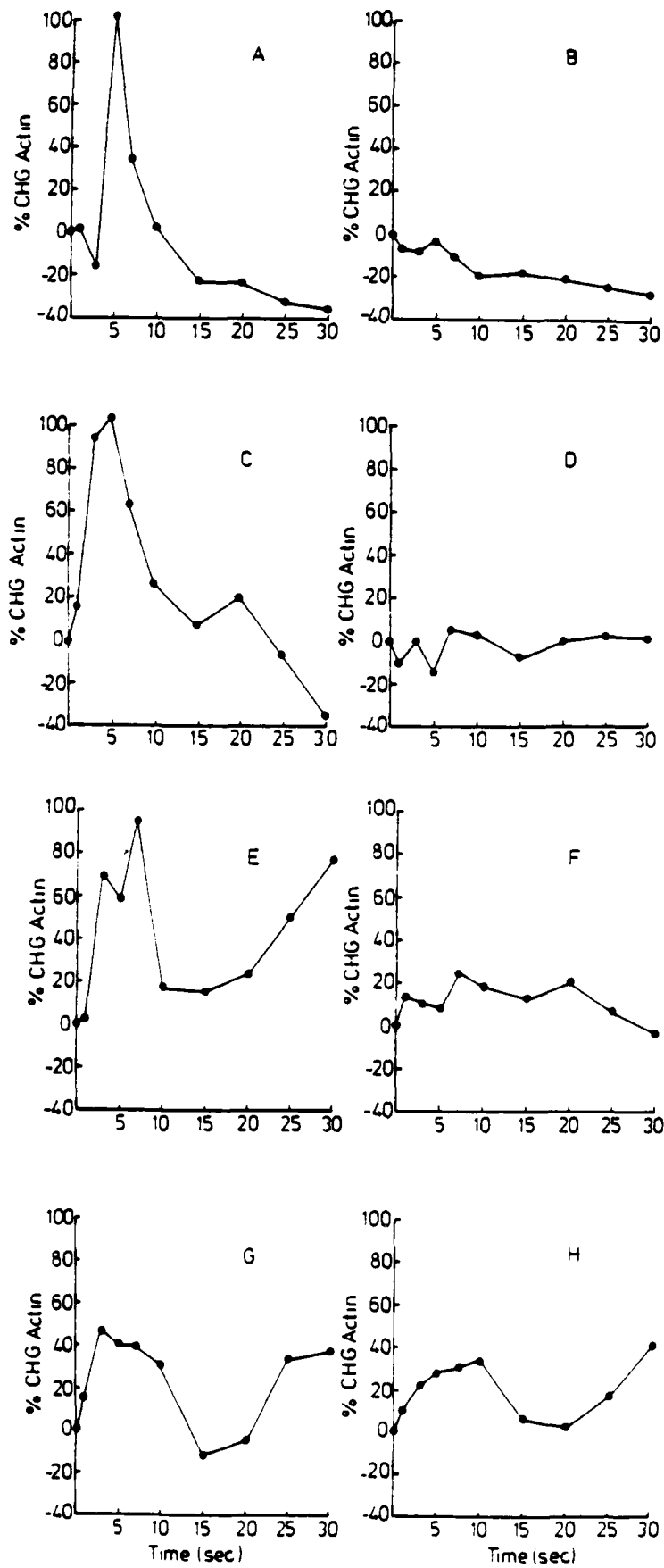


Fig. 10.1

Figure 10.1    The Effect of Various TMB-8 Concentrations on the Actin Response

D. discoideum cells, after 12hr of development in shaker culture, were suspended at  $10^8$  cells  $\text{ml}^{-1}$ , incubated for 40min with or without TMB-8, then stimulated with folate (50 $\mu\text{M}$ ). Triton insoluble cytoskeletons were prepared at preset times (0-30sec) after folate stimulation. Actin content was determined by one dimensional SDS-PAGE and scanning densitometry. Actin content is expressed as percent change in actin from the 0sec basal value. Data for each TMB-8 concentration is from a single experiment.

- a) 12 hr control cells incubated 40 min
- b) 12 hr cells incubated 40 min  
with 2mM TMB-8
- c) 12 hr control cells incubated 40 min
- d) 12 hr cells incubated 40 min  
with 1.5mM TMB-8
- e) 12 hr control cells incubated 40 min
- f) 12 hr cells incubated 40 min  
with 1.0mM TMB-8
- g) 12 hr control cells incubated 40 min
- h) 12 hr cells incubated 40 min  
with 0.75mM TMB-8

## 10.2 Results

### A Effects of TMB-8 on the 12-Hour Actin Response

Dictyostelium discoideum cells of strain NC4 were allowed to develop for 12hr in shaker suspensions and then washed and resuspended at  $10^8$  cells  $\text{ml}^{-1}$  in calcium-free SORPHOS. Aliquots (3ml) of this cell suspension were then incubated with additions of TMB-8, or TMB-8 plus  $\text{CaCl}_2$ , at various concentrations for 40min on a rotary shaker (170rpm). Cells were subsequently pelleted by centrifugation and washed once in 10ml calcium-free SORPHOS and resuspended at  $10^8$  cells  $\text{ml}^{-1}$  for use in the folate-stimulated actin response assay.

The effects of various concentrations (0.75mM-2mM) of TMB-8 on the actin response are shown in figure 10.1. The actin response was partially inhibited with 0.75mM TMB-8 and 1mM TMB-8 was the minimum concentration required to inhibit the actin response (figure 10.1 and 10.2). Treatment with 2mM TMB-8 caused some cell lysis, observed as turbidity in the supernatant after the 40min treatment period, and microscopic analysis showed minor perturbations in membrane conformation. However, centrifugation through silicone oil at a density known to exclude lysed cells confirmed that the majority of 2mM TMB-8 treated cells were not lysed.

### B Effect of Extracellular Calcium on TMB-8 Inhibition of the Actin Response

Vegetative cells were harvested, allowed to develop for 12hr in shaker culture and then incubated with TMB-8 (0.75mM and 1.0mM) or with TMB-8 plus  $\text{CaCl}_2$  (10mM). Results of these experiments appear in figure 10.3. The results for 0.75mM TMB-8 are those presented in section A above with the addition of the  $\text{CaCl}_2$  effect. The effect of 1mM TMB-8, shown above to be the minimum concentration necessary to block the actin

responses, presumably by replenishing internal stores. cAMP receptor binding was not affected by TMB-8 which suggests that its inhibitory action on aggregation and cGMP accumulation occurs at a location between the cell surface receptors and the guanylate cyclase enzyme. The conclusion that intracellular calcium stores are the source of essential  $\text{Ca}^{2+}$  is further supported by observations that EGTA treatment (similar to that used for TMB-8) does not inhibit cGMP accumulation (Mato et al., 1977; Europe-Finner and Newell, 1984) and a report that the calcium ionophore A23187 can enhance the cGMP response in the absence of extracellular  $\text{Ca}^{2+}$  (Brenner and Thoms, 1984) presumably by redistributing the intracellular  $\text{Ca}^{2+}$  (Borle, 1978; Feinstein, 1978).

To determine if calcium was an essential messenger in signal transduction leading to tip formation, I studied the effects of TMB-8 on the actin response. Preliminary data from McRobbie (1984) had shown reduced recoveries of cytoskeletal actin after TMB-8 treatment of aggregation competent cells (6-8hr) and an inability to protect the actin response with additions of extracellular calcium. However, the actin assays were conducted with an extracellular  $\text{CaCl}_2$  concentration of 7mM and it has been shown that even much lower  $\text{Ca}^{2+}$  levels significantly disrupt cytoskeletal actin extractions (Rosenberg et al., 1981). These early problems were overcome in this study and results of the effects of TMB-8 and calcium on the 12hr actin response are reported here.

During the course of this work, the drug TMB-8 was implicated as a strong inhibitor of the electron transport chain in thymocytes (Brand and Felber, 1984). To confirm this effect in D. discoideum, cell respiration rates were measured after amoebae had been pre-treated with TMB-8 or TMB-8 plus  $\text{CaCl}_2$ .

## 10 CALCIUM MOBILIZATION MAY BE REQUIRED FOR THE FOLATE-INITIATED ACTIN RESPONSE

### 10.1 Introduction

Cytoplasmic calcium may be a second messenger linking occupied cell surface receptors with the intracellular mechanisms involved in cell movement. Stimulation of D. discoideum amoebal suspensions with high concentrations of cGMP or physiological concentrations of cAMP or folate, induces a transient influx of  $^{45}\text{Ca}^{2+}$  from the extracellular medium within 6sec which peaks at 30sec (Wick et al., 1978; Bumann et al., 1984). However, the literature, though somewhat contradictory, suggests that extracellular calcium levels may not significantly affect cell responsiveness. Mason et al. (1971) and Malchow et al. (1982) have reported that EGTA inhibits aggregation and chemotaxis while others (Mato et al., 1977; Saito, 1979) found that extracellular EGTA had no effect on aggregation. Europe-Finner and Newell (1984) found that EGTA does inhibit aggregation but only after prolonged exposure at high concentrations; they proposed that such treatment depleted intracellular  $\text{Ca}^{2+}$  stores and thereby blocked cell movement. Devreotes (1982) also suggested an intracellular role for calcium and proposed that Dictyostelium might have mechanisms for controlling cytoplasmic calcium levels in diverse environments.

To further characterize the internal  $\text{Ca}^{2+}$  role in cell aggregation, Europe-Finner and Newell (1984) used TMB-8 as an inhibitor of intracellular calcium mobilization. This compound has been reported to have such an effect in several other cell types (Malagodi and Chiou, 1974; Gorman et al., 1979; Owen and Villereal, 1982). Europe-Finner and Newell (1984) found that 1-2mM TMB-8 inhibited aggregation and cAMP mediated intracellular cGMP accumulation in D. discoideum. Addition of extracellular  $\text{Ca}^{2+}$  (4-7mM) restored the inhibited aggregation and cGMP

## *Chapter 10*

responses at the tipped aggregate stage.

In summary, I was unable to demonstrate that the folate-induced actin response corresponding with tip formation involves signal transduction or cell movement. However, the results of this chapter do support the signal transduction model of McRobbie and Newell (1984b).

responses and temporally elongated migration periods during aggregation (Ross and Newell, 1981). Initially, his results were perhaps confusing as well. The "streamer" mutants showed no elevated actin responses after cAMP stimulation but, instead, demonstrated responses identical to the wild-type (McRobbie and Newell, 1984b). Additionally, their work showed an additive effect on the actin response when 3hr dual-responsive cells were simultaneously stimulated with folate and cAMP. In a similar experiment studying cGMP responses, Van Haastert (1983) had shown no such additive effect on cGMP responses. This discrepancy, plus the "streamer" result, suggested that cGMP did not control the actin response and prompted McRobbie and Newell to propose an altered model for chemotactic signal transduction in Dictyostelium discoideum (McRobbie and Newell, 1984b; figure 1.3a).

As suggested by the model, if cGMP does not mediate the actin response then an elevated cGMP response at 12hr of development may not occur. Consequently, the negative result described in this chapter would be explicable. An alternative explanation is suggested by the report of Schaap and Wang (1985) published after the completion of this work. They reported a small rise in cGMP levels, from 0.3 pmoles per  $10^7$  cells to 0.6 pmoles per  $10^7$  cells, when early culmination stage aggregates (16hr) of D. minutum were stimulated with cAMP. Additionally, they reported that this small cGMP response was lost if the morphological integrity of the culminants was not carefully preserved when developing aggregates were harvested. The cGMP assay, as employed in my study, did not have the sensitivity necessary to detect a small cGMP response such as the one described by Schaap and Wang (1985). Also, in my study, the developing aggregates were harvested by vigorous vortexing to disrupt the aggregates and thereby expose maximum cell surface area and maximum numbers of cell surface receptors. Again, this experimental approach may have prevented the detection of weak cGMP

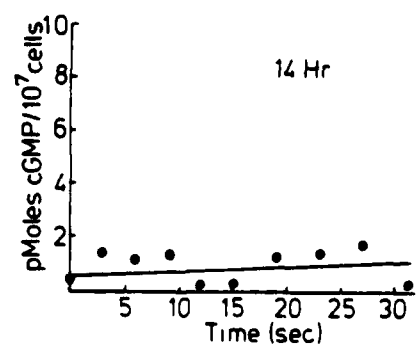
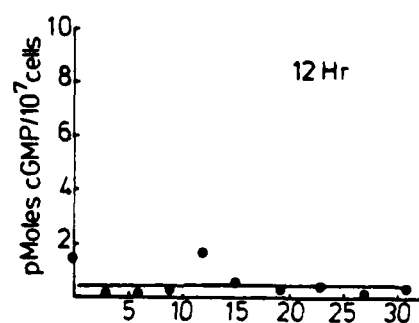
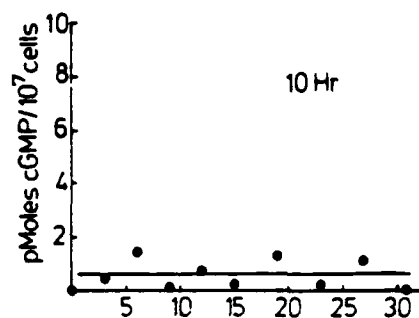
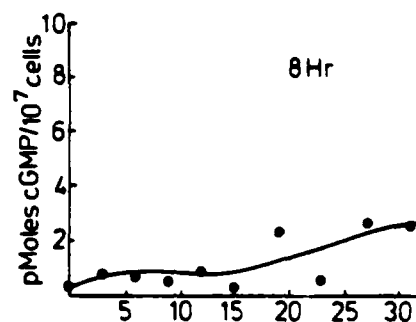
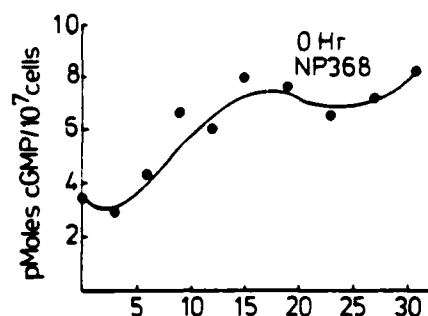


Fig 9.2

Figure 9.2    The Folate-elicited cGMP Response during  
"Streamer" Mutant Development

Washed vegetative amoebae were allowed to develop for 0-14hr on millipore filters and then suspended at  $10^8$  cell ml<sup>-1</sup> for the cGMP assay. Cells were stimulated with folate (50μM) and then lysed at preset times (0-31sec) with perchloric acid (1.6% v/v). Supernatants were analysed for cGMP content by radioimmunoassay. These data are means from two separate experiments.

## B Folate-Stimulated cGMP Responses During Development of a "Streamer" Mutant

The "streamer" mutant, NP368, from complementation group stmF (stmF406 in Ross and Newell, 1981) demonstrates significantly elevated cGMP levels after cAMP stimulation (3-fold greater than wild-type) due to lack of cGMP-specific phosphodiesterase (Coukell et al., 1984). Therefore, this mutant was employed to study its folate-induced cGMP responses during development. If the 10-14hr cGMP responses were present but very weak in the wild-type (perhaps below assay sensitivity), it seemed feasible that the responses might be elevated in strain NP368 and therefore more easily detected. Figure 9.2 summarizes data from two experiments and shows the characteristic elevated cGMP response at 0hr (cf. figure 9.1); no significant cGMP responses were evident at 10-14hr of development.

### 9.3 Discussion

Folate-induced cGMP responses were routinely detected in vegetative cells but the cGMP response was not evoked in cells from 10-14hr aggregates. Additionally, a "streamer" mutant, NP368, known to express elevated cGMP responses upon ligand stimulation was employed to attempt to obtain an enhanced cGMP response at 10-14hrs. However, renewed cGMP responses later in development, potentially indicative of signal transduction and cell movement, were not detected in this mutant. This suggested that the actin response at 12hr of development did not involve the cGMP response and perhaps did not involve signal transduction related to cell movement.

Initially, this was a disappointing finding. However, during the course of this study, McRobbie in our lab studied cAMP-induced actin responses in two "streamer" mutants (NP368 and NP377). He anticipated an enhanced actin response corresponding with the elevated cGMP

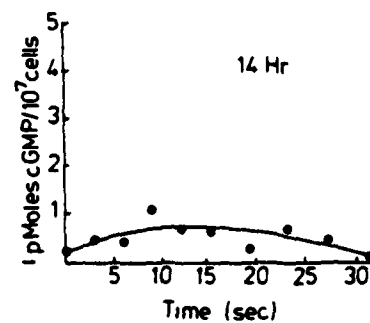
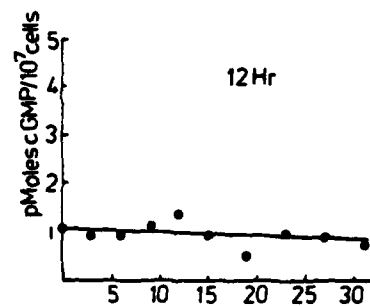
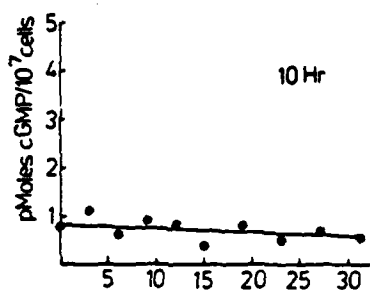
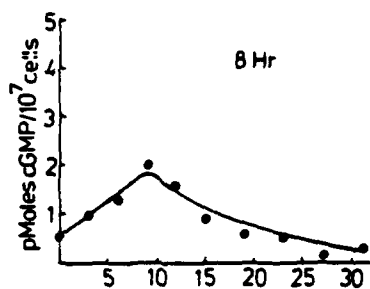
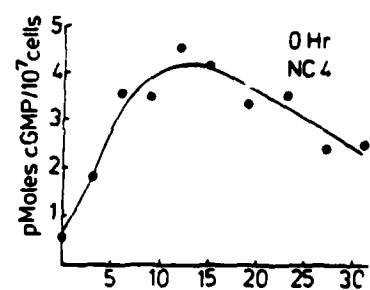


Fig 9.1

Figure 9.1    The Folate-elicited cGMP Response during  
Wild-type NC4 Development

Washed vegetative amoebae were allowed to develop for 0-14hr on Millipore filters and then suspended at  $10^8$  cells  $\text{ml}^{-1}$  for the cGMP assay. Cells were stimulated at preset times (0-31sec) with folate ( $50\mu\text{M}$ ) and then lysed with perchloric acid (1.6% v/v). Supernatants were analysed for cGMP content by radioimmunoassay. These data are means from three separate experiments.

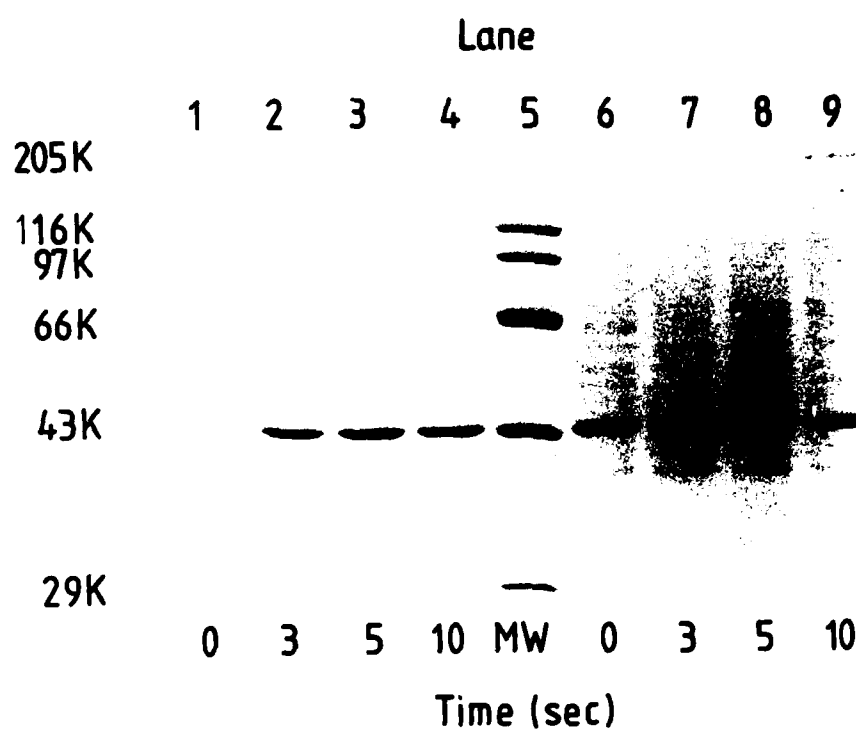


Fig. 10 .2

Figure 10.3 Effect of Extracellular Calcium on TMB-8  
Inhibition of the Actin Response

D. discoideum cells, allowed to develop 12hr in shaker culture, were resuspended at  $10^8$  cells  $\text{ml}^{-1}$ , incubated for 40min with or without TMB-8, then stimulated with folate ( $50\mu\text{M}$ ). Cytoskeletons were prepared and the actin content measured as in figure 10.1. Each set of data is from a single experiment.

- a) 12 hr control cells
- b) 12 hr cells +  $1\text{mM}$  TMB-8
- c) 12 hr cells +  $1\text{mM}$  TMB-8 +  $10\text{mM}$   $\text{CaCl}_2$
- d) 12 hr control cells
- e) 12 hr cells +  $0.75\text{mM}$  TMB-8
- f) 12 hr cells +  $0.75\text{mM}$  TMB-8 +  $10\text{mM}$   $\text{CaCl}_2$

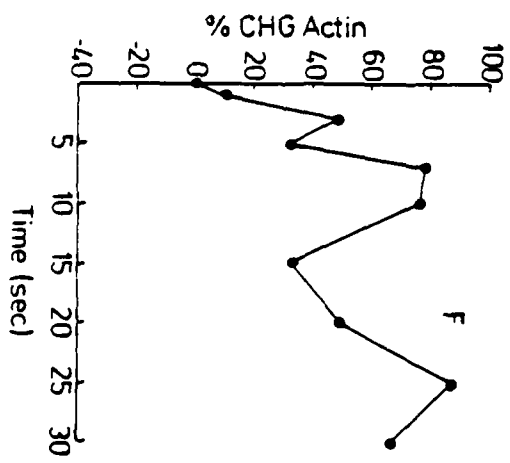
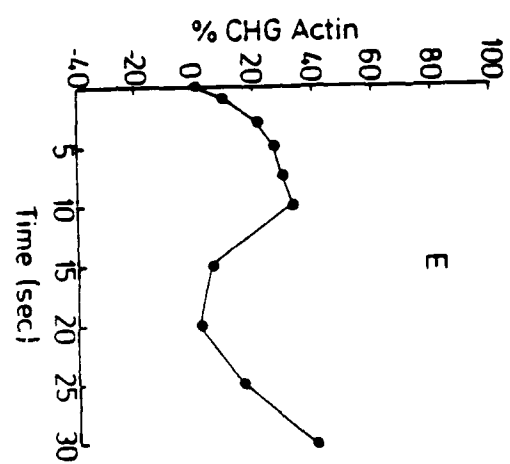
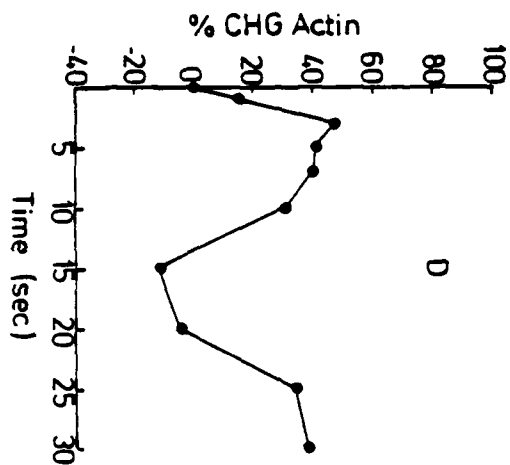
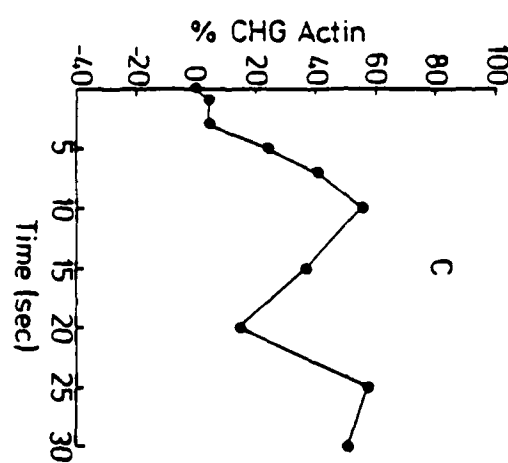
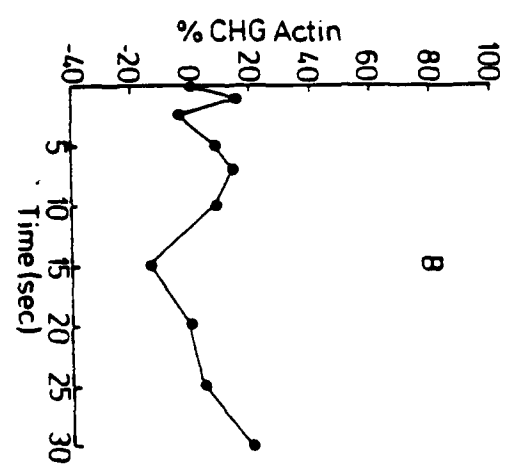
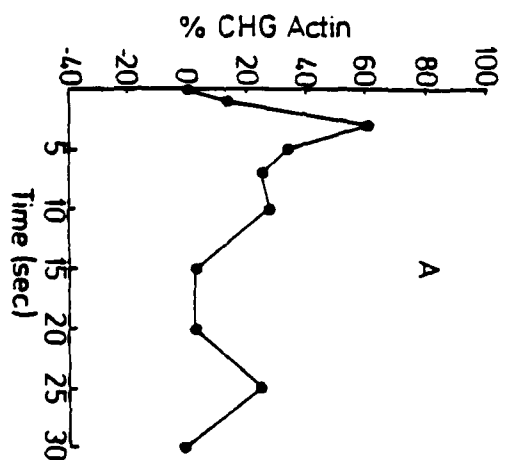


Fig 10.3

response, was abolished in the presence of 10mM  $\text{CaCl}_2$ . Although 0.75mM TMB-8 did not fully inhibit the actin response, added  $\text{CaCl}_2$  seemed to enhance this response in the presence of TMB-8.

#### C Effect of TMB-8 on Cellular Respiration in D. discoideum

Cell suspensions of  $2-5 \times 10^7$  cells  $\text{ml}^{-1}$  in SORPHOS were transferred to a 10ml water-jacketed ( $25^\circ\text{C}$ ) Clark type oxygen electrode constructed by N.A.P. Wood (Wood, 1978). The rate of oxygen consumption was measured by the change in electrical potential. The zero and 100% settings for the electrode were established as described in chapter 2. Air-saturated buffer at  $25^\circ\text{C}$  contained 240  $\mu\text{moles O}_2$  per litre (Chappell, 1964); therefore, it was possible to calculate oxygen consumption directly from the recorder trace.

Cells were treated with increasing concentrations of TMB-8 by accumulative additions of 0-100  $\mu\text{M}$  TMB-8 during 30min and respiration rates measured after each stepwise addition of TMB-8. Fresh cells were treated in an identical manner during a second 30min period to determine the effect of 50  $\mu\text{M}$ -200  $\mu\text{M}$  TMB-8 on cellular respiration. The combined results of these two experiments are shown in figure 10.4. TMB-8 at a concentration of 150  $\mu\text{M}$  caused maximal inhibition of respiration and reduced the cell respiration rate to 30% of the rate for untreated control cells. TMB-8 concentrations above 150  $\mu\text{M}$  showed no further inhibition of respiration. The maximal dose of 150  $\mu\text{M}$  could possibly be an overestimate since the effect of incubation time on the TMB-8 effect was not controlled in this experiment.

#### D Effect of Rotenone, Succinate, and Calcium on TMB-8-Inhibited Cell Respiration

Control cells were treated with the electron-transport inhibitor, rotenone (20  $\mu\text{M}$ ), or rotenone followed by succinate (6mM) to determine

Figure 10.4 Effect of TMB-8 on Cellular Respiration in  
Vegetative Amoebae

Vegetative amoebae, washed and resuspended at  $5 \times 10^7$  cells  $\text{ml}^{-1}$  in 10ml SORPHOS, were placed in a water-jacketed  $\text{O}_2$  electrode reservoir at  $25^\circ\text{C}$ .  $\text{O}_2$  consumption rates were measured at 3-5min intervals after stepwise accumulative additions of 0-200 $\mu\text{M}$  TMB-8. The 2mM TMB-8 value is the respiration rate after 40min incubation. These results are from a single experiment.

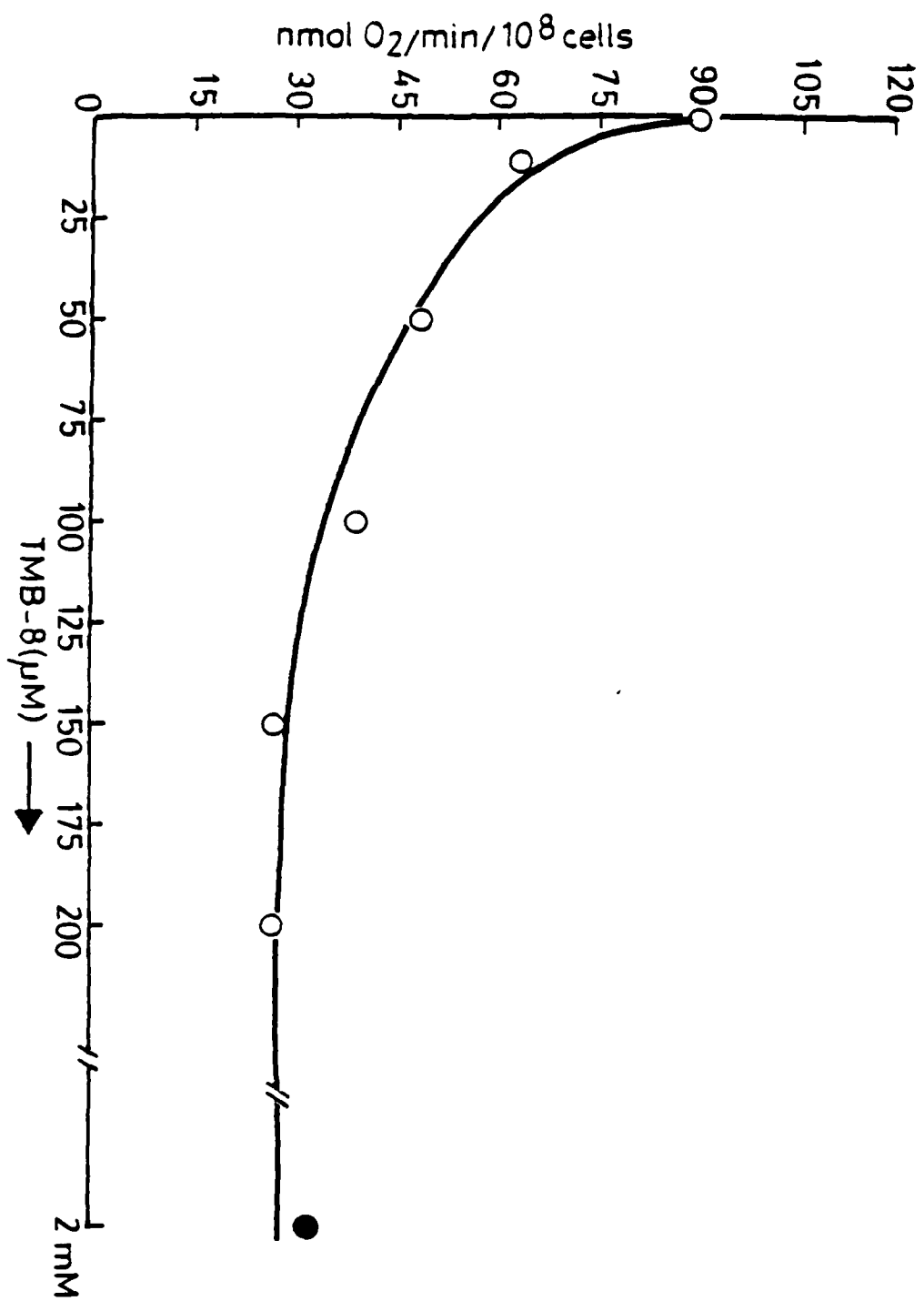


Fig. 10.4

the effect of blocking ATP Site I with rotenone and to see if succinate would overcome this block. Rotenone inhibited respiration to 39% of control levels and succinate metabolism, which bypasses this site I block, restored respiration to 69% of the control rate (table 10.1 and figure 10.5).

Respiration rates were measured after treatment of cells with three concentrations of TMB-8 alone or with either succinate or calcium. Incubation for 40min in TMB-8 reduced respiration to 30% of the control rate (table 10.1, figure 10.5). The effect of succinate on TMB-8 inhibition of respiration was determined by adding succinate after the 40min TMB-8 treatment period. Succinate provided partial recovery for TMB-8 treated (200 $\mu$ M, 1mM, 2mM) cells and allowed cells to respire at 50% of the control rate. Cells treated with TMB-8 plus calcium were provided significant protection and respired at 55-80% of the control levels. An even greater effect was seen when succinate was added to cells that had previously been incubated with TMB-8 plus  $\text{CaCl}_2$  for 40min, suggesting that calcium and succinate may function in a synergistic manner to stimulate respiration (table 10.1, figure 10.5).

The effects of an extended range of calcium concentrations on TMB-8-inhibited respiration was examined in another experiment. Vegetative amoebae were harvested, washed and resuspended at  $2 \times 10^7$  cells  $\text{ml}^{-1}$  in calcium-free SORPHOS. After measurement of control respiration levels, TMB-8 was added to a final concentration of 200 $\mu$ M and respiration again measured after 5min incubation. Subsequently, the same cells in 200 $\mu$ M TMB-8 were treated with increasing concentrations of  $\text{CaCl}_2$  by accumulative additions during 30min.

After the 5min incubation in 200 $\mu$ M TMB-8, cellular respiration declined to 40% of control levels and this level of inhibition was unchanged during the accumulative addition of 3mM  $\text{CaCl}_2$ . However, at 5mM  $\text{CaCl}_2$ , oxygen consumption was 80% restored and further calcium

Table 10.1

The Effect of TMB-8 on Respiration in D. discoideum Amoebae

<u>Cell Treatment</u> *	<u>% of Control Respiration</u>
Control	100%
Control + 6mM Succinate	100%
Rotenone (20uM)	39%
Rotenone (20uM) + 6mM Succinate	69%
TMB-8 (200uM),	31%
TMB-8 (200uM) + 6mM Succinate	47%
TMB-8 (200uM) + 7mM CaCl <sub>2</sub>	81%
TMB-8 (200uM) + 6mM Succinate + 7mM CaCl <sub>2</sub>	114%
TMB-8 (1mM)	22%
TMB-8 (1mM) + 6mM Succinate	46%
TMB-8 (1mM) + 7mM CaCl <sub>2</sub>	77%
TMB-8 (1mM) + 6mM Succinate + 7mM CaCl <sub>2</sub>	108%
TMB-8 (2mM)	22%
TMB-8 (2mM) + 6mM Succinate	47%
TMB-8 (2mM) + 7mM CaCl <sub>2</sub>	55%
TMB-8 (2mM) + 6mM Succinate + 7mM CaCl <sub>2</sub>	92%

\* In the tests with TMB-8, amoebae were incubated for 40min with TMB-8 and with or without calcium at the concentrations indicated prior to addition of succinate and prior to measurement of O<sub>2</sub> consumption. These results are from a single experiment and are representative of data from several experiments.

Figure 10.5 Representative Traces from O<sub>2</sub> Electrode Experiments

Vegetative amoebae were harvested, washed, and resuspended at  $3 \times 10^7$  cells  $\text{ml}^{-1}$  in SORPHOS. A series of cell samples were incubated with or without TMB-8 (200 $\mu\text{M}$ , 1 $\text{mM}$ , 2 $\text{mM}$ ) or with TMB-8 plus 7 $\text{mM}$   $\text{CaCl}_2$  and incubated for 40min. Oxygen consumption rates of the treated cells were measured with an oxygen electrode. Traces a), b), c) show the effect of TMB-8 after 40min treatment (.....).

(X) represents measurement of O<sub>2</sub> consumption under various conditions:

(——) - TMB-8 (40min) plus 6 $\text{mM}$  succinate added at time of respiration measurement

(----) - 7 $\text{mM}$   $\text{CaCl}_2$  also present during TMB-8 treatment period

(---) - 7 $\text{mM}$   $\text{CaCl}_2$  also present during TMB-8 treatment period and 6 $\text{mM}$  added at time of respiration measurement

Trace d) shows the effect of 20 $\mu\text{M}$  rotenone injected while measuring O<sub>2</sub> consumption, and the effect of subsequent injection of 6 $\text{mM}$  succinate. O<sub>2</sub> consumption rates have been normalized to a standard control rate to allow ready comparison of treatment effects.

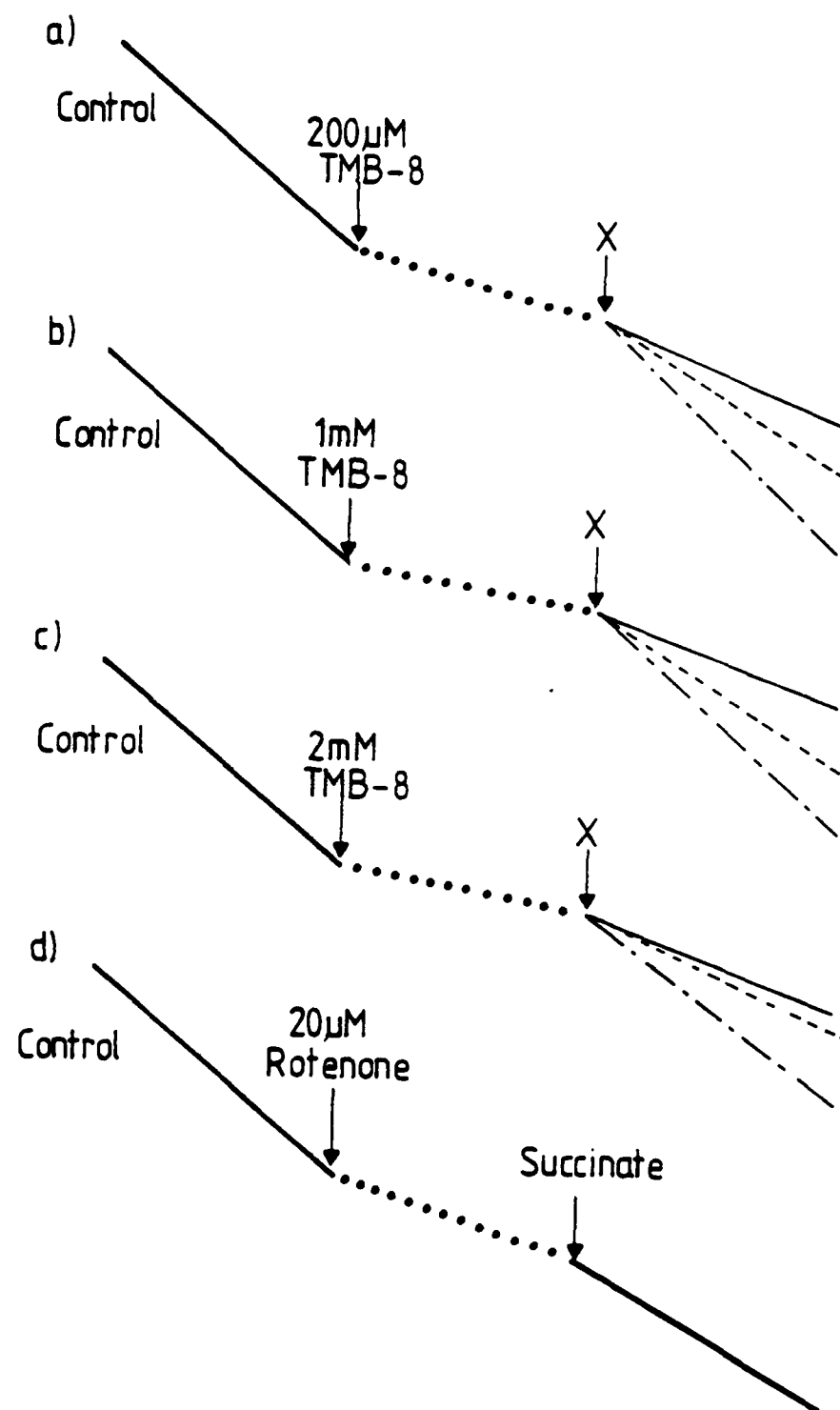


Fig 10.5

Figure 10.6    Effect of Calcium on TMB-8-treated  
Vegetative Amoebae

Vegetative amoebae, washed and resuspended at  $2 \times 10^7$  cells  $\text{ml}^{-1}$  in 10ml SORPHOS, were placed in a water-jacketed  $\text{O}_2$  electrode reservoir at  $25^\circ\text{C}$  and then  $\text{O}_2$  consumption was measured prior to TMB-8 addition (unstimulated), after  $200\mu\text{M}$  TMB-8 plus accumulative additions of  $\text{CaCl}_2$  ( $\circ$ ), or  $200\mu\text{M}$  TMB-8 plus  $\text{CaCl}_2$  and  $6\text{mM}$  succinate ( $\bullet$ ). The dotted line (----) traces the oscillations of the respiration level after succinate additions. These data are from a single experiment.

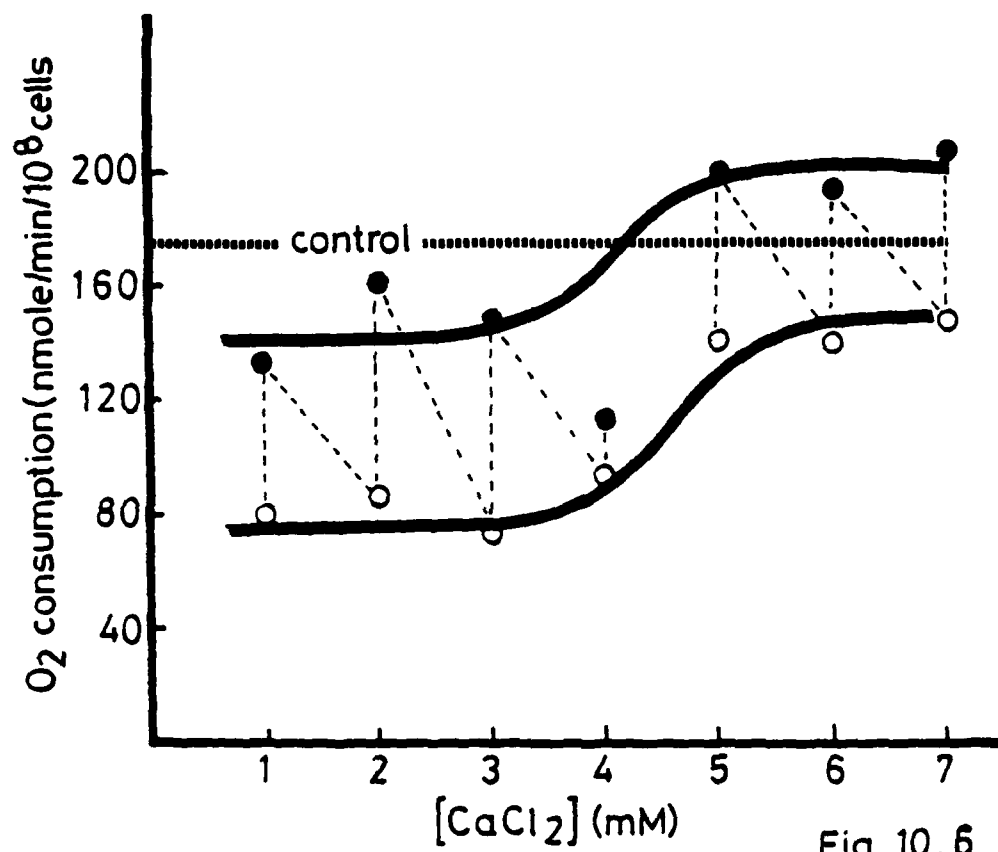


Fig 10.6

additions had no additional stimulatory effect on respiration (figure 10.6). During this experiment sufficient succinate was added after each calcium addition to provide a final concentration of 6mM and respiration was again measured. It was evident from the oxygen consumption measurements that the succinate was rapidly degraded since respiration levels returned to presuccinate levels during each subsequent reoxygenation step. The succinate additions further enhanced the cell respiration rate and at extracellular calcium levels above 4mM the respiration rate exceeded the level for control cells (figure 10.6, also evident in table 10.1).

### 10.3 Discussion

The folate-induced actin response was strongly inhibited by 1mM TMB-8 yet a nearly normal actin response occurred if  $\text{CaCl}_2$  (10mM) was also present (figure 10.1, 10.3). Therefore, as seen for aggregation and cGMP responses (Europe-Finner and Newell, 1984), calcium provided protection from the inhibitory effects of TMB-8. These parallel results suggest that calcium plays an important role in signal transduction and possibly folate-induced cell movement at 12hr of development. However, TMB-8 also strongly inhibits cell respiration in D. discoideum; therefore, any calcium protective effects must be interpreted with caution.

The cellular respiration experiments with vegetative cells showed that after 40min treatment with 200 $\mu\text{M}$ , 1mM or 2mM TMB-8, the  $\text{O}_2$  consumption rates were inhibited to 25% of control rates. If 6mM succinate was added after the 40min drug treatment, respiration rates were restored to 50% of control respiration levels. The protection provided by succinate to TMB-8 treated cells was similar to the recovery effect seen when rotenone-treated cells were administered succinate; in both cases the added succinate approximately doubled the  $\text{O}_2$  consumption

rate (table 10.1). TMB-8 appears to be a potent inhibitor of cellular respiration in D. discoideum and the similar effect of succinate on rotenone and TMB-8 treated cells suggests that TMB-8 may act at the site proposed by Brand and Felber (1984) (i.e. NADH dehydrogenase).

Interestingly, additions of extracellular calcium in the presence of TMB-8 stimulated respiration rates to an even greater degree than succinate (table 10.1, figure 10.6). Furthermore, succinate and  $\text{CaCl}_2$ , when combined, seemed to stimulate cellular respiration in a synergistic manner (table 10.1, figure 10.6).

The concentration of calcium found to stimulate cellular respiration is quite similar to the concentration required to recover ligand-induced responses. Calcium at 5-7mM restores cellular respiration to 75% of control levels (200 $\mu\text{M}$  and 1mM TMB-8 treated cells) and 7-10mM  $\text{CaCl}_2$  is sufficient to recover the cellular responses of aggregation (1-2mM TMB-8), cGMP accumulation (2mM TMB-8) (Europe-Finner and Newell, 1984) and cytoskeletal actin accumulation (1mM TMB-8) (figure 10.3). In comparison, the level of TMB-8 required to inhibit cellular responses (1-2mM) is 10-fold greater than that required to inhibit cellular respiration (100-150 $\mu\text{M}$ ).

Several interpretations of these results are possible. If the block in cellular responses caused by TMB-8 is due entirely to the reduced level of respiration in the treated cells, then 200 $\mu\text{M}$  TMB-8 should be sufficient to inhibit the cellular responses. The fact that 10-fold higher concentrations of TMB-8 are required suggests that TMB-8 may be functioning in a dual role. TMB-8 may immobilize  $\text{Ca}^{2+}$  and thereby block cellular responses when administered at high concentrations (1-2mM) and this effect may be distinct from the respiration inhibition effect. This would suggest that ATP generation may not be critical for these cellular responses.

Figure 11.6 Contact Sites A Development  
in the "Brainy" Mutant

Vegetative amoebae of strains NC4 (—) and NP550 (#####) were allowed to develop in shaker culture and cells were analyzed at various times (0-12hr) for the appearance of contact sites A (EDTA resistant adhesion sites). Degree of cell aggregation was recorded as percent of all cells that remained as single cells. These are data from a single experiment.

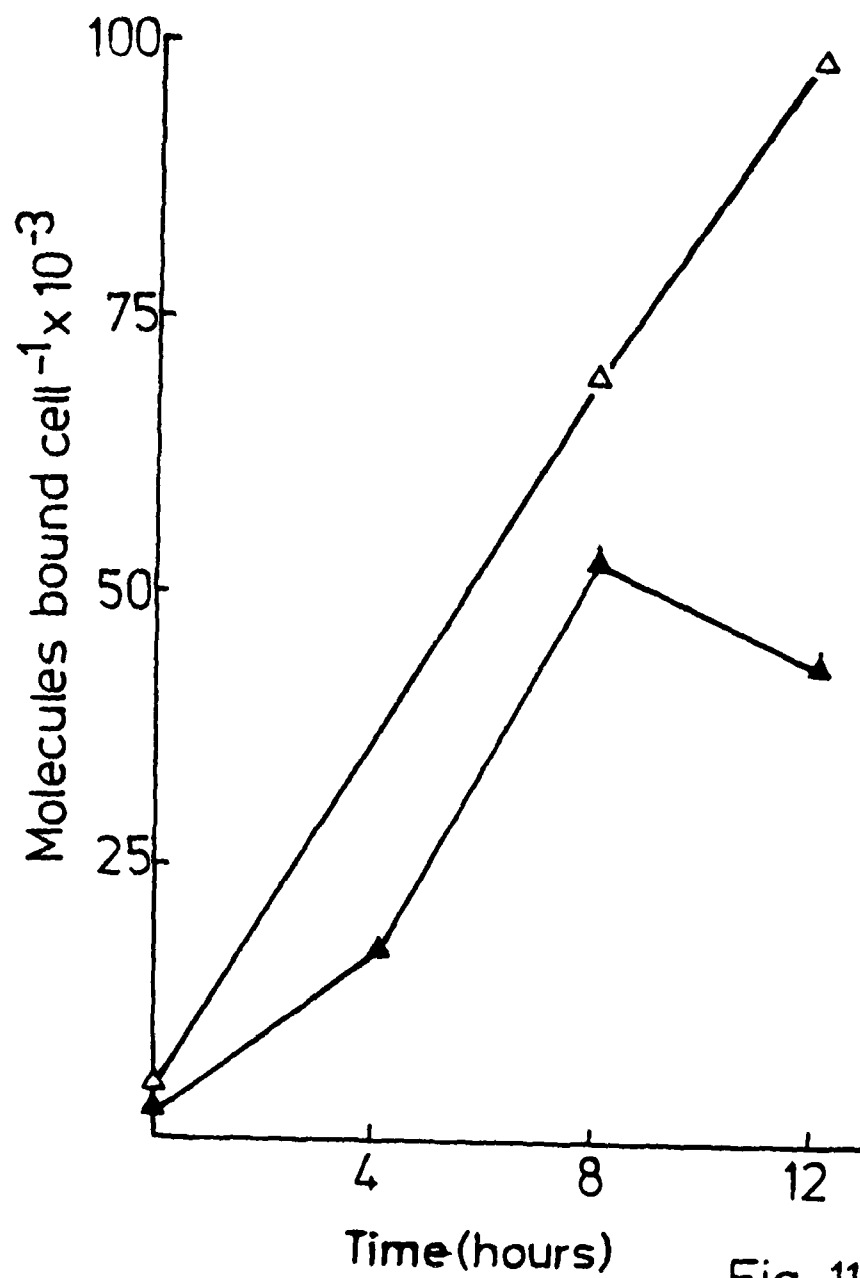


Fig 11.5

Figure 11.5 Cell-surface cAMP Receptors on the  
"Brainy" Mutant during Development

[<sup>3</sup>H]cAMP binding to D. discoideum strains NC4  
( △ ) and NP550 ( ▲ ) during development in  
shaker culture was measured at 200nM  
[<sup>3</sup>H]cAMP. These data are from a single  
experiment.

of decline of receptors on NP550 may be delayed compared to wild-type NC4, perhaps reflecting delayed development in NP550. However, the developmental regulation of folate receptors on NP550 was quite comparable to that seen in NC4.

#### C Cell-Surface cAMP Receptors on NP550 during Development

Vegetative amoebae of strains NC4 and NP550 were allowed to develop in shaker culture and then analysed at various times of development for the appearance of cAMP receptors. The cAMP receptors on the surface of NP550, detected with 200nM [<sup>3</sup>H]cAMP, increased from background levels to 53,000 sites by 8hr of development and then declined to 43,000 by 12hr (figure 11.5). The wild-type, NC4, showed a similar receptor increase by 8hr but a decline comparable to that seen for NP550 was not evident. The decline in receptors after 8hr of development as seen in NP550 more accurately fits published data which showed a decline in receptor density after 10hr of development (Green and Newell, 1975).

#### D Appearance of Contact Sites A during NP550 Development

Vegetative amoebae of strains NP550 and NC4 were allowed to develop in shaker culture for various times and then analysed for the appearance of contact sites A as previously described (chapter 2). Contact sites A are defined as EDTA resistant cell adhesion sites (Gerisch, 1961). In both the wild-type cells and the "brainy" mutant, EDTA resistant adhesion sites were detected by 8hr of development (figure 11.6). The data are expressed as percent single cells present after EDTA treatment; at least 200 cells were scored at each time point. Although the onset of contact sites A development may be somewhat delayed in NP550, the mutant appeared to develop normal cell cohesion sites by 12hr of development.

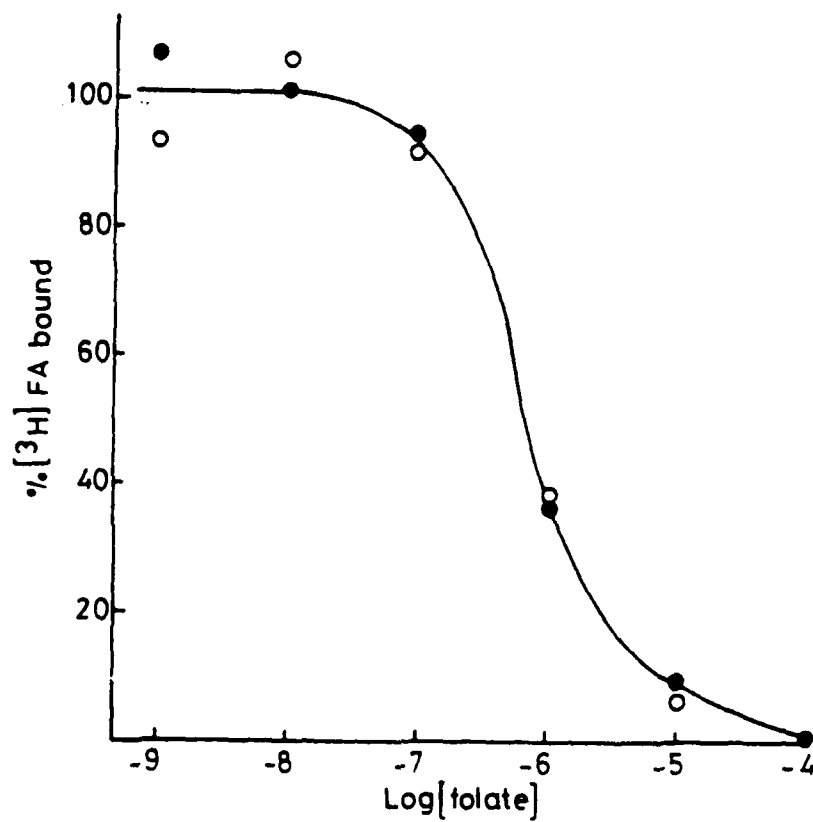


Fig 11.3

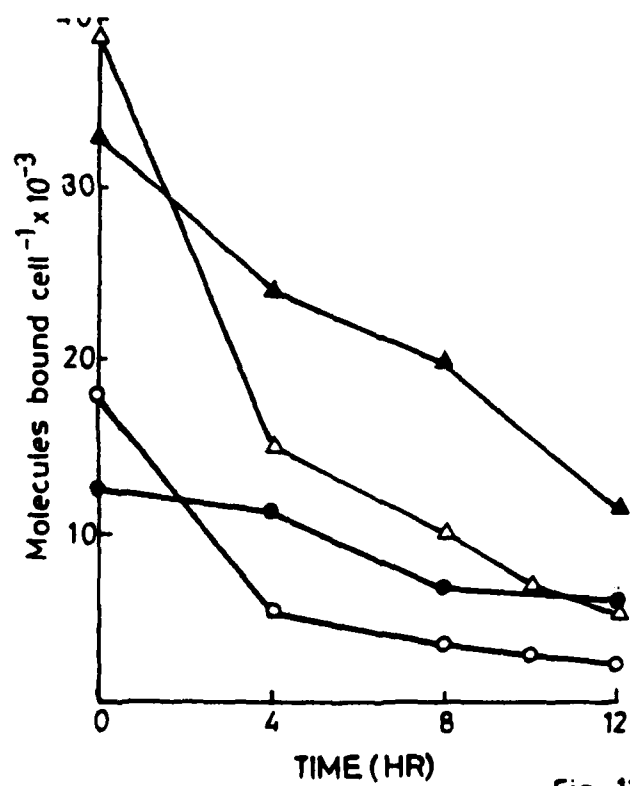


Fig. 11.4

Figure 11.3     Cell-Surface Folate Receptors  
on the "Brainy" Mutant

Vegetative amoebae of D. discoideum, strains NC4 (●) and NP550 (○) were incubated with 300nM [<sup>3</sup>H]folate, various concentrations of unlabelled folate ( $10^{-9}$ - $10^{-4}$ M), and 8-azaguanine (0.5mM). This result is from a single experiment.

Figure 11.4     Developmental Regulation of Cell-surface  
Folate Receptors on the "Brainy" Mutant

[<sup>3</sup>H]folate binding to D. discoideum strains NC4 (open symbols) and NP550 (closed symbols), after development in shaker culture for various times (0-12hr), was measured at 500nM (△ ▲) and 100nM (○ ●). These data are from a single experiment.

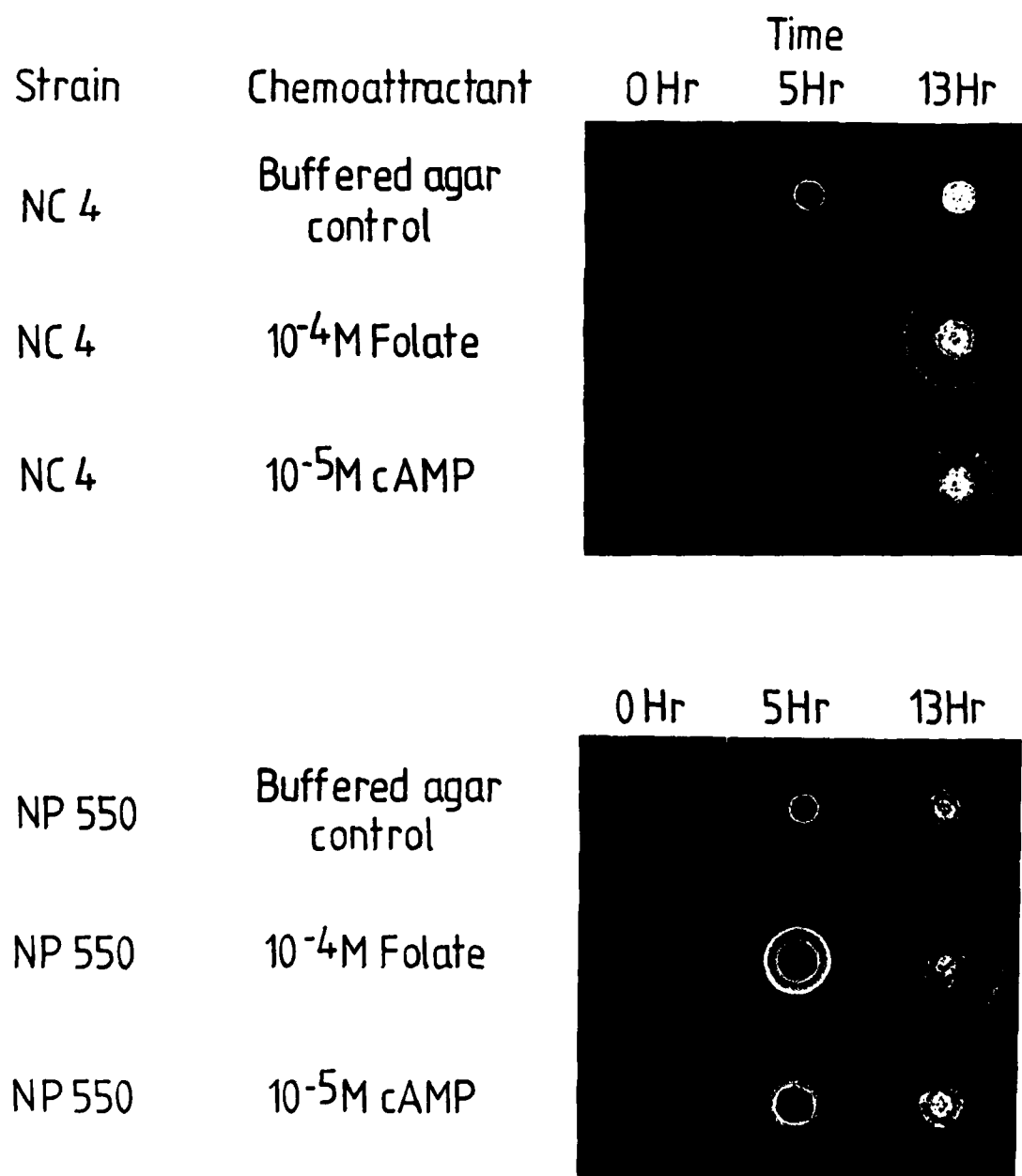


Fig 11.2

Figure 11.2    Chemotaxis of the "Brainy" Mutant

The chemottractants cAMP and folate were mixed with with 2% Oxoid agar in SORPHOS. Vegetative amoebae of strains NC4 and NP550 were washed clear of bacteria and then resuspended as a dense slurry (approx.  $10^9$  cells  $\text{ml}^{-1}$ ). One microlitre cell aliquots were spotted onto the agar surface, allowed to dry briefly, and incubated at  $22^\circ\text{C}$  in a humidified chamber. Degree of chemotaxis was recorded by photography through dark field optics after 5 and 13hr incubation.

- a) Strain NC4
- b) Strain NP550

To determine competence the following characteristics were assessed: chemotaxis towards folate and cAMP, presence of folate and cAMP receptors, and formation of contact sites A.

A Folate- and cAMP-Induced Chemotaxis by the "Brainy" Mutant, NP550

Chemotaxis towards folate and cAMP was determined by the "spreading" assay. Vegetative amoebae of NP550 and the parent wild-type NC4 were harvested and washed clear of bacteria then spotted onto non-nutrient 2% Oxoid agar in SORPHOS with the appropriate chemoattractant uniformly dispersed through the agar at various concentrations. The degree of chemotactic movement was recorded by photography through dark-field optics and the results are presented in figure 11.2. The "brainy" mutant NP550 appeared to display normal chemotaxis towards folate and cAMP.

B Cell-Surface Folate Receptors on NP550 during Development

Vegetative NP550 amoebae were initially assayed for the presence of folate receptors by the folate competition binding assay (chapter 5). Vegetative NP550 amoebae seemed to possess characteristic folate binding receptors and an  $I_{50}$  of approximately 600nM, nearly identical to that seen for folate receptors on NC4 control cells in this single experiment (figure 11.3).

The developmental regulation of folate receptors was also evaluated after allowing NP550 cells to develop for various times in shaker culture. The density of cell-surface folate receptors on NP550 cells declined during development (figure 11.4) with a pattern of regulation similar to that seen in the wild-type NC4 (cf. figure 4.1, Tillinghast and Newell, 1984). Data for NC4 folate binding were extracted from chapter 4, figure 4.1 and are included here for comparison (figure 11.4). The initial density of receptors on NP550 appeared, in this single experiment, to be somewhat lower than the wild-type and the rate

A



B



C



Fig. 11.1

Figure 11.1 Comparison of the Wild-type and  
"Brainy" Mutant Phenotypes

Wild-type and "brainy" mutant clones on mass  
plates (Magnification approx. 5X)

- a) Wild-type, strain NC4
- b) "Brainy" Mutant, NP550
- c) Fully developed "Brainy" aggregates

## 11 CHARACTERIZATION OF A DEVELOPMENTAL MUTANT: THE "BRAINY" MUTANT

### 11.1 Introduction

In this section of the thesis, I describe studies of the role of cell-to-cell contact in tipped aggregate formation and slime mould development. By comparing various characteristics of a developmental mutant of D. discoideum with the wild-type strain and by studying cell-to-cell interactions of wild-type and mutant cells, it was possible to gain a further understanding of essential cell-to-cell contact events that may occur during slime mould development. The roles of cell-cell contact and cell interactions in development were reviewed in chapter 1.

I employed a class of mutants that are able to develop to the tight aggregate stage (chapter 1) but do not form tipped aggregates and do not complete culmination. This group of mutants was isolated in this lab after NTG mutagenesis of wild-type strains XP55 and NC4 and was labelled "brainy" mutants to describe the phenotype during aggregation. Instead of the slightly wavy surface seen during aggregation of the wild-type on clearing mass plates, these mutants display a very convoluted surface quite similar to the cerebral cortex of the human brain, hence, the name "brainy" (figure 11.1). Although several members from the mutant group were employed at the outset of this study, the only member characterized in detail was NP550 from the wild-type parent strain NC4. This mutant had not been previously characterized or used in any past studies.

### 11.2 Results

#### 11.2.1 Development of Aggregation Competence in the "Brainy" Mutant

The mutant, NP550, was first evaluated for its ability to attain aggregation competence. It seemed possible that, although the mutant proceeded beyond the aggregation stage of development, the "brainy" phenotype may have reflected an abnormality in aggregation competence.

## *Chapter 11*

This first interpretation, however, must be approached with caution. Added calcium may allow cellular responses (i.e. aggregation, cGMP accumulation, actin accumulation) to occur in TMB-8 treated cells because of the substantial increase in respiration rate that occurs with added calcium. This interpretation would suggest that TMB-8 blocks respiration in two ways: one, via NADH dehydrogenase which can be bypassed if succinate is supplied as a substrate and, two, by immobilizing calcium and thereby reducing the essential calcium needed for normal electron-transport function. The synergistic stimulation of respiration seen when both succinate and calcium (figure 10.6) are added to TMB-8 treated cells (200 $\mu$ M-1mM) indicates that this may be true.

In light of the complex effects of TMB-8 on respiration, it is impossible to distinguish between the effects of TMB-8 on calcium mobilization related to mitochondrial function and those related to cellular response. In fact, it appears that TMB-8 inhibition of the cellular responses related to cell movement, aggregation and the actin response, may be entirely due to inhibition of respiration. No correlation between the actin response at 12hr and the role of calcium in signal transduction is possible from these results. Therefore, to demonstrate that the folate-induced actin response at 12hr is mediated by signal transduction will require the application of an alternative approach in a later study.

The oxygen electrode data presented in this chapter were obtained during collaboration with Dr. G.N. Europe-Finner, Biochemistry Department, Oxford University.

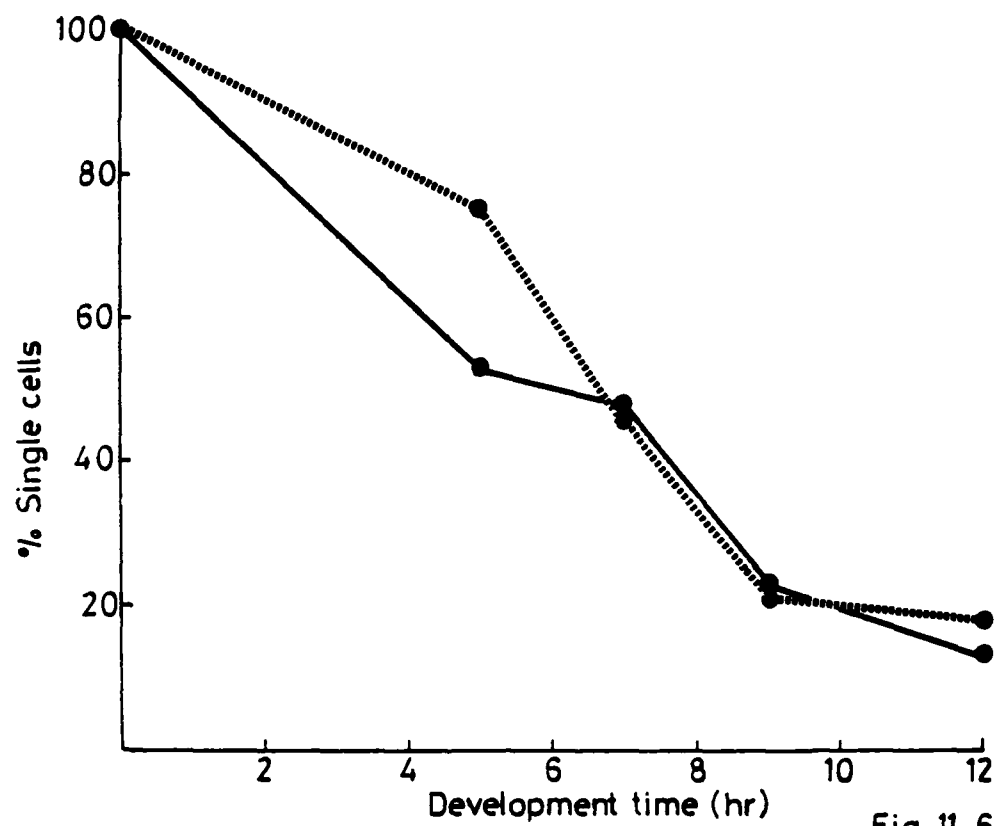


Fig 11.6

Table 11.1

Appearance of Pre-spore Vesicles  
during "Brainy" Mutant Development

Development Time (hr)	Strain	Pre-Spore Vesicle Presence
0	NC4	-
16	NC4 Slugs	++
0	NP550	-
24	NP550	-
30	NP550	-
36	NP550	-
42	NP550	+/-
44	NP550	+
48	NP550	++
50	NP550	++

- = No fluorescent pre-spore vesicles detected.

+/- = Fluorescence localized around nucleus.

+ = Fluorescent pre-spore vesicles in greater than 50% of the cells.

++ = Fluorescent pre-spore vesicles in greater than 90% of the cells.

### 11.2.2 Prespore and Prestalk Specific Proteins Produced by the "Brainy" Mutant

Prespore and prestalk specific proteins are first synthesized at the tipped aggregate stage of development, and protein synthesis accumulation is most prominent in the migrating slugs and during early culmination (Morrissey *et al.*, 1984). Prespore cells contain cell-type specific prespore vesicles (PVs) and cell-type specific antigens, present on the surface of these vesicles, are later found on the surface of mature spores (Takeuchi, 1963; Devine *et al.*, 1983). By using two-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) as well as polyspecific and monoclonal antibodies, workers have identified a number of cell-type specific proteins (Alton and Brenner, 1979; Morrissey *et al.* 1981, 1984; Ratner and Borth, 1983; Tasaka *et al.* 1983; Wallace *et al.* 1984).

Developing cells of the "brainy" mutant were analysed for the appearance of prespore vesicles and for the appearance of cell-type specific proteins. The prespore vesicle anti-serum (anti-PV) was obtained from Dr. James Morrissey and was absorbed with vegetative amoebae of strain Ax3 prior to use.

#### A Prespore Vesicles Produced by the "Brainy" Mutant

Vegetative amoebae of stain NP550 were allowed to develop on non-nutrient agar (KMMP) and then harvested at various times of development and tested for the presence of prespore vesicles. For PV analysis, cells were fixed, incubated with anti-PV antiserum (rabbit), and then with rhodamine-conjugated swine anti-rabbit serum. Through 40hr of development, no PVs were evident; however, between 40 and 50hr the majority (approx. 90%) of the developing mutant cells acquired prespore vesicles (table 11.1, figure 11.7). The specificity of the anti-serum was verified by analysis of front (prestalk) and rear (prespore) cells

Figure 11.7     Fluorescent Microscopy of Prespore Vesicles

- a) NC4 Prestalk Cells - Cells from the front one-fifth of migrating slugs were stained with anti-spore serum (rabbit) and TRITC-conjugated swine anti-rabbit serum and photographed through a Leitz epifluorescence microscope.
- b) NC4 Prespore Cells - Cells from the central three-fifths of migrating slugs were stained and photographed as in a).
- c) NP550 Cells - Cells from 53hr developing aggregates were stained and photographed as described in a).

These photographs are representative examples from six separate experiments.

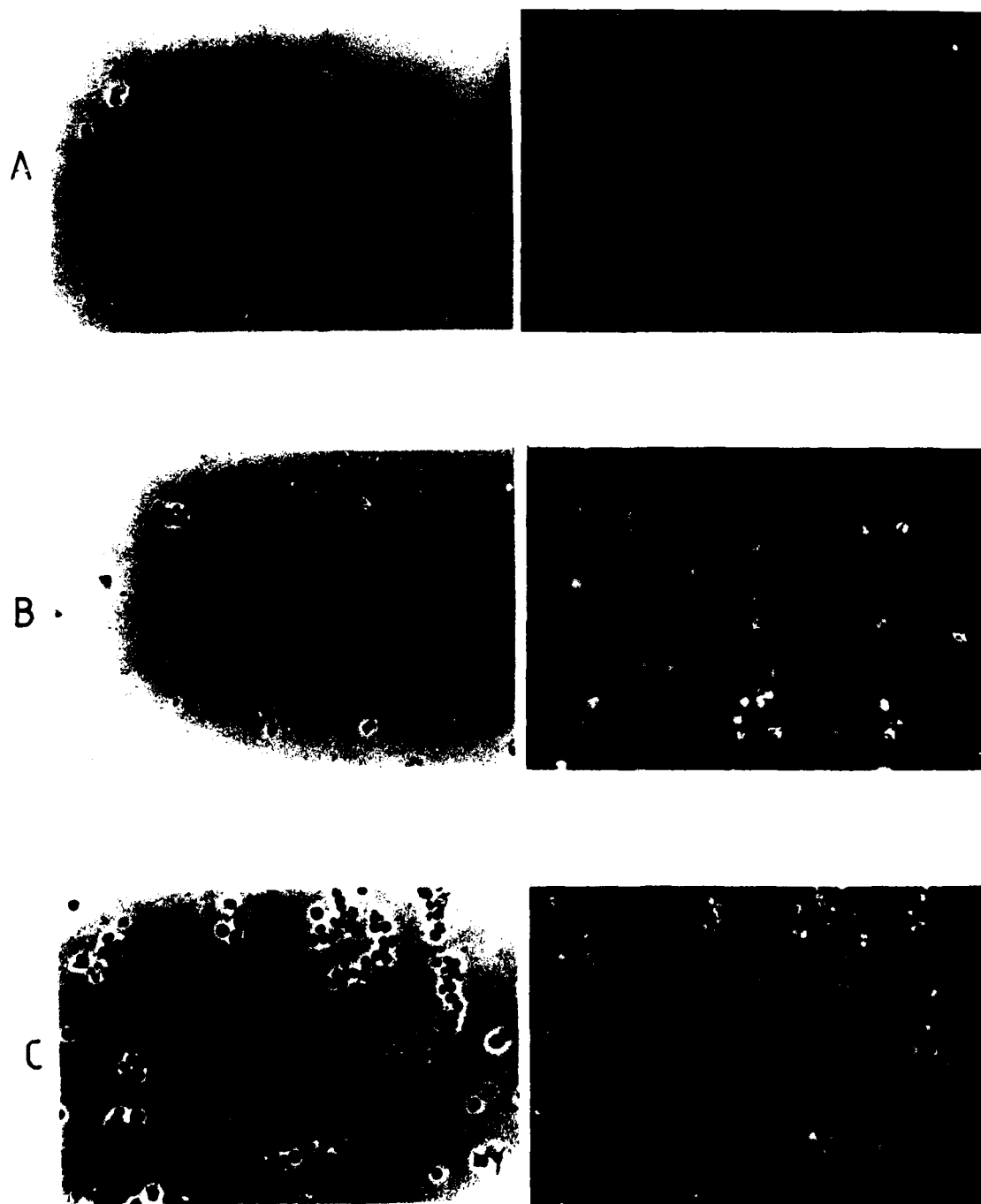


Fig. 11.6

Table 11.2

Pre-spore and Spore-specific Proteins  
from "Brainy" Mutant Aggregates

Pre-spore and Spore Identifiers *	45-50 Hour NP550
Psp 29	-
33a	+
33b	+
40	-
59	+
127	+
130	+
150	-
SP 27	+
28a	+
29a	+
29b	+
60	+
70	-
96	+
210	+

\* (Morrissey et.al., 1984)

from migrating wild-type slugs of strain NC4 for the presence of PVs. The low percentage front cells with PVs relative to the rear cells verified the specificity of the anti-serum (figure 11.7).

#### B 2D-NEPHGE of Developing Cells from the "Brainy" Mutant

Vegetative amoebae of strain NP550 were developed on Millipore filters and, at various times of development, cells were harvested and cell samples were prepared for 2D-NEPHGE (chapter 2). Two dimensional gels were analysed for the appearance of cell-type specific proteins by comparing the protein pattern with a reference gel which showed the complete series of cell-type specific proteins previously identified in spores and whole slugs (Morrissey et al. 1984) (figure 11.8). A series of prespore and spore specific proteins were detected in 45-50hr NP550 aggregates which corresponded temporally with the appearance of prespore vesicles (tables 11.1, figure 11.8). The prespore and spore specific proteins detected are listed in table 11.2 and are compared to those identified by Morrissey et al. (1984). There is a significant delay in the appearance of cell-type specific proteins in the mutant. The protein pattern seen in mutant cells at 24hr (figure 11.8e) is similar to that seen in wild-type cells after only 4hr of development (figure 11.8c). A prominent protein in the 24hr mutant cell sample is gp80, the putative contact site A glycoprotein, thus further verifying that the mutant does produce contact sites A during development.

#### C Discussion

From the results of the chemotaxis tests, the folate and cAMP binding studies, and the contact sites A experiment the "brainy" mutant was considered fully able to become aggregation competent.

The "brainy" mutant NP550 showed significantly delayed development; however, at 45-50hr prespore-specific protein markers were detected, verifying that the mutant possessed the genetic ability to make spore

Figure 11.8 Prespore- and Spore-specific Proteins from  
"Brainy" Mutant Aggregates Identified by 2D-NEPHGE

Prespore proteins from developing amoebae and spore proteins from mature fruiting bodies were separated by 2D-NEPHGE. Photographs A,B were kindly supplied by Dr. James H. Morrissey (Morrissey, et al., 1984) to provide a comparison for photographs C-F.

- a) Spore proteins, silver stained
- b) Spore proteins, <sup>35</sup>S autoradiography
- c) 4 hr wild-type cells, silver staining
- d) 24 hr wild-type fruiting bodies, silver staining
- e) 24 hr NP550 cells, silver staining
- f) 53 hr NP550 cells, silver staining

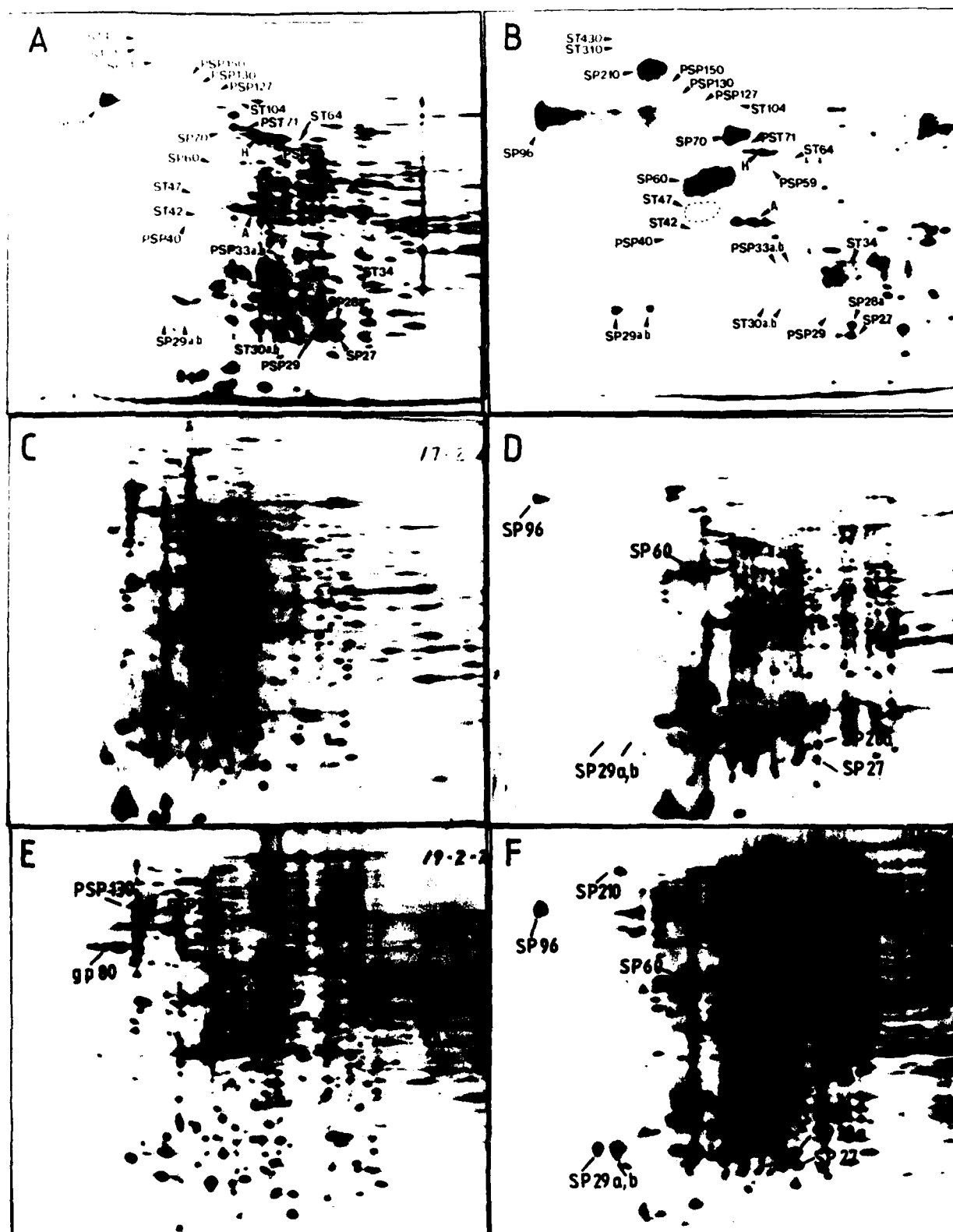


Fig 11.8

and prespore specific proteins. Prespore vesicles, characteristic of prespore cells appeared at 50hr of development and a series of five prespore-specific and seven spore-specific proteins were identified at this time of development by 2D-NEPHGE. The anti-PV serum was produced by injection of D. mucoroides spores and therefore one would expect the surface antigens detected on prespore vesicles to be those found on the surface of mature spores. Devine et al. (1983) have confirmed that several spore coat proteins (sp60, sp70, sp96) are packaged in prespore vesicles.

This immunological and 2D-NEPHGE study of the NP550 developing aggregates confirmed that NP550 is significantly delayed in development and requires nearly three times longer than the wild-type to produce spore specific proteins. At the time of spore protein production (53hr), the morphology of the "brainy" aggregate is a hemispherical aggregate without a tip, but the mound is somewhat compressed at the base, suggesting that the aggregate may be attempting to rise off the substratum. Microscopic examination of cells from this morphological stage showed no mature spores or spore-like cells (data not shown).

From these results, it is tempting to suggest that the "brainy" mutant is capable of producing prespore type cells but is unable to produce prestalk cells since tips are not produced on the developing aggregates. Based on this assumption, studies involving altered environmental conditions and synergy studies with wild-type cells were conducted to evaluate the potential of this mutant for studying cell-to-cell contact interactions and prespore differentiation.

## *Chapter 12*

## 12 DEVELOPMENT OF NP551 UNDER VARIOUS ENVIRONMENTAL CONDITIONS AND DURING SYNERGISTIC ASSOCIATION WITH WILD-TYPE CELLS

### 12.1 Introduction

Past workers have induced developmental mutants to revert to the wild-type phenotype or to produce predominately one cell type by altering the environmental conditions (Weber and Raper, 1971; Darmon et al., 1975; Kay et al., 1978; Gross et al., 1983; Kay and Jermyn, 1983). Consequently, in this study, the "brainy" mutant was exposed to a broad spectrum of environmental conditions in an attempt to induce more advanced development. In addition to the original "brainy" mutant, NP550, a methanol-resistant isolate, strain NP551, was used in the synergy studies.

Inducement of mutant development by synergistic interactions with wild-type or other mutant cells has often been employed to obtain viable mutant spores for laboratory storage, thereby ensuring prolonged immortality of the mutant strain. Furthermore, several workers have employed synergy to study cell-to-cell contact and development. Sussman (1955) studied the synergistic interactions of a variety of aggregateless mutants and showed for many combinations that two aggregateless mutants were able to complement each other and induce the development of mature fruiting bodies and mature viable spores of both mutant types. In each of these successful synergistic interactions, the mature spores produced clones that displayed only one or the other of the original phenotypes, thereby suggesting that syngamy or syngensis was not occurring and hybrid spores were not being produced. Sussman (1955) suggested as alternatives that syntrophism, where biochemical intermediates were shared, may have occurred or that one or the other of the two cell types (prestalk/prespore) was missing and cell differentiation and fruiting body construction could not occur unless

the alternate cell-type was made available through synergy. Rafaeli (1962) described synergy in two developmentally defective Polysphondylium violaceum mutant strains and also reported no evidence of recombinant spores (i.e., clones from spores retained either one or the other original phenotype). Rafaeli suggests that synergism may occur through anastomosis and temporary cell fusions where exchange of cytoplasm would allow mutants to develop in synergy, however, he did not exclude the possibility of molecular exchange through intact plasma membranes.

Bonner and Adams (1958) amputated tips from developing wild-type aggregates, constructed interspecies chimeras and then observed the effects on development. In some cases they observed segregation of cell types, in others double fruiting bodies formed (one on top of the other), and a third case showed complete mixing of cell types. Filosa (1962) suggested that heterocytosis (mixtures of genotypically distinct individuals) may exist in slime mould aggregates in the natural environment. He proposed that such a heterogeneous state may be a way for haploid organisms to control variation as it provides evolutionary plasticity, equivalent to storing of recessives in homologous chromosomes of diploid organisms or in haploid nuclei of heterokaryons. Atryzek (1976) constructed chimeras from two D. discoideum strains with significantly different rates of development and observed that synergistic interactions altered timing of spore formation in both mutants. Other workers mixed different slime mould species and genera to study interspecies cell-to-cell adhesion (Nicol and Garrod, 1978).

I conducted wild-type and "brainy" mutant synergy experiments and determined the effects of synergy on the terminal development of the "brainy" mutant. Additionally, membrane barrier experiments were conducted to determine the need for cell-to-cell contact on inducement of mutant development.

## 12.2 Results

### 12.2.1 Effects of Various Environmental Conditions on "Brainy" Mutant Development

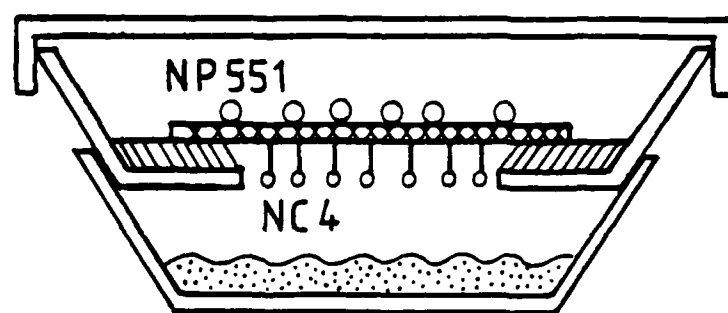
For this series of experiments, NP550 cells were allowed to develop on Millipore filters over buffer or chemical-soaked pads, or on filters over a buffered agar surface.

#### A "Brainy" Mutant Development in the Absence of a Bacterial Food-Source

The "brainy" phenotype was most evident when the mutant cells were allowed to develop in the presence of the bacterial food source. It seemed possible that development might proceed to a greater degree in the absence of the bacterial metabolites. Such an effect has been described for other D. discoideum mutants (Weber and Raper, 1971). However, the earlier receptor experiments and cell-specific protein experiments had allowed development to occur under starvation conditions without any evident advance in terminal development. Further experiments are described here that attempted to induce complete development in a starvation environment.

Mutants may become blocked in development as a result of abnormal accumulations of inhibitory metabolites (Sussman et al., 1978). To test this possibility in NP550, mutant cells were washed free of bacteria and transferred to the surface of Millipore or Nucleopore filters over lower pad solution (LPS) soaked pads (Sussman and Lovgren, 1965) or over KMMP agar. Lids of the petri plates were lined with an additional pad soaked in upper pad solution (UPS) (Newell et al., 1969). The altered environment provided by the UPS, designed to trap excess ammonia, had no effect on mutant development.

Cyclic AMP has frequently been implicated as the chemoattractant that attracts tip cells and induces tip formation (Sussman, 1982). If






-  1% Sorphos buffered agar
-  Agar - coated nucleopore filter
-  Sorphos buffer

Fig. 12.2

Figure 12.2    Suspended Membrane Experiment

Vegetative cells of NC4 and NP551 were washed and resuspended at  $1 \times 10^8$  cells  $\text{ml}^{-1}$  then 0.1ml samples of each cell type were deposited to opposite sides of the suspended membrane. Development was allowed to proceed for 48hr.

were unable to develop when restricted in a monolayer under the filter barrier (Kay et al., 1978) and therefore did not produce a "diffusible factor" essential for NP551 development. However, as seen for large pore filters, if NC4 was able to develop on top of the filter barrier and perhaps produce the "diffusible factor" then NP551 was also induced to develop.

To test this hypothesis, an apparatus was designed that included a suspended membrane thus allowing development of NC4 and NP551 on opposite sides of the suspended membrane. The apparatus consisted of two 50mm petri plates with a 20mm hole punched in the top plate. A thin film of 1% agar in SORPHOS was poured around the upper hole. To form the membrane, a 0.6µm Nucleopore filter, 25mm, was dipped in molten SORPHOS buffered agar and immediately laid over the 20mm hole, allowing it to seal against the thin agar film (figure 12.2). I thought that if NC4 developed to culmination and produced the "diffusible factor", the factor could diffuse across the agar coated membrane and induce NP550 development.

An equivalent number of NC4 and NP551 vegetative amoebae ( $1 \times 10^7$  cells) were plated onto opposite sides of the suspended membrane and allowed to develop. NC4 developed to normal culmination in 24hr but NP551 showed no enhanced development. Therefore, either NC4 does not induce NP551 via a "diffusible factor" or the membrane barrier was too thick to allow sufficiently high concentrations of the factor to reach the NP551 mutant aggregates.

### 12.3 Discussion

The "brainy" mutant, strain NP551, did not develop beyond the hemispherical aggregate stage, despite attempts to induce development under a wide spectrum of environmental conditions. Since the "brainy" mutant did not develop tipped aggregates, it was suspected that the

from wild-type cells could induce mutant development, wild-type and mutant cells were separated by a variety of membranes and development was allowed to proceed. For these experiments, monolayer lawns of one strain were formed on SORPHOS buffered agar and then filters, coated on the top surface with the other test strain, were placed over the partially dried monolayer. The cells were then allowed to develop for 48hr in a humidified chamber. Spores were harvested with a sterile Nichrome wire loop and treated with 0.1% NP-40 as described previously.

Two varieties of two different types of filters served as membrane barriers: Millipore filters, 25mm in diameter and 150 $\mu$ m thick, with either 1.2 $\mu$ m or 0.45 $\mu$ m pores; Nucleopore filters, 25mm in diameter and 10 $\mu$ m thick, with either 2.0 $\mu$ m or 0.60 $\mu$ m pores. As shown in table 12.2, only when the pores in the filter membrane barrier were sufficiently large to allow direct cell-to-cell contact and physical mixing of cell types did the wild-type induce development of the "brainy" mutant. The 0.45 and 0.60 $\mu$ m pores of the Millipore and Nucleopore filters, respectively, excluded transmembrane migration; therefore, with these filters, mutant culmination through synergy did not occur.

In control experiments with NC4 in the lawn and no cells on the overlay filter, wild-type culminants developed on the filter surface of the filters with 1.2 $\mu$ m and 2.0 $\mu$ m pores. Therefore, it was evident that transmembrane migration of the strains and cell-to-cell contact did occur, resulting in synergistic development of the "brainy" mutant. Fruiting bodies containing mutant spores formed when the mutant was in the lawn. This showed that the mutant was also able to transit the large pore filters.

#### H NC4-Induced Development of NP551 Across a Suspended Membrane

It seemed possible that the reason NC4 was unable to induce development of NP551 through small pore filters was because NC4 cells

Table 12.2

Strain on Agar	Strain on Filter over Agar	Type * Filter	Development (48 hr)	% Mutant ** Spores
NC4	NP551	2.0 $\mu$ N	Culmination	37%
NC4	NP551	0.6 $\mu$ N	No Culmination	—
NC4	—	2.0 $\mu$ N	Culmination	0%
NC4	—	0.6 $\mu$ N	No Culmination	—
NC4	NP551	1.2 $\mu$ M	Culmination	34%
NC4	NP551	0.45 $\mu$ M	No Culmination	—
NC4	—	1.2 $\mu$ M	Culmination	0%
NP551	NP551	2.0 $\mu$ N	No Culmination	—
NP551	NC4	2.0 $\mu$ N	Culmination	26%
NP551	NC4	0.6 $\mu$ N	Culmination	0%
NP551	—	2.0 $\mu$ N	Aggregation <sup>++</sup>	0%
NP551	—	0.6 $\mu$ N	No Aggregation <sup>£</sup>	—
NP551	NP551	1.2 $\mu$ M	No Culmination	0%
NP551	NC4	1.2 $\mu$ M	Culmination	32%
NP551	NC4	0.45 $\mu$ M	Culmination	0%
NP551	—	1.2 $\mu$ M	Aggregation <sup>++</sup>	0%
NP551	—	0.45 $\mu$ M	No Aggregation <sup>£</sup>	—

\* N = Nucleopore, M = Millipore

\*\* Data are means from two separate experiments.

++ NP551 cells migrated through the membrane.

£ No NP551 cells on the filter surface.

The "brainy" mutant was not induced to culminate when incubated with non-viable NC4 cells or membrane fragments. Cell surface wild-type membrane components alone were apparently ineffective in inducing mutant development.

E Effect of Wild-Type Cell Extracts on "Brainy" Mutant Development

"Brainy" mutant cells were allowed to develop in the presence of wild-type cell extracts in the form of conditioned media or the sterilized extract from sonicated NC4 cells that had developed for 15hr. The conditioned media was obtained by starving NC4 amoebae in shaker culture ( $2 \times 10^7$  cells  $\text{ml}^{-1}$  in SORPHOS) for 24hr. For the sonicated cell extract, cells that had developed for 15hr in shaker culture were sonicated and the mixture was then filter sterilized. This experiment was an attempt to mimic the proposed anastomosis effect (Rafaeli, 1962) by exposing mutant cells to the secretions from, or the cytoplasm of, wild-type cells. These wild-type extracts failed to induce further NP551 development.

F Grafting of the Prestalk and Prespore Regions of Wild-type Slugs to NP551 Tight Aggregates

The prestalk (tip) and prespore (back) zones from wild-type slugs, developed on 2% (w/v) unbuffered Noble agar, were grafted to NP551 tight aggregates previously developed on KMMP agar. After 48hr of development, the resulting fruiting bodies were analysed for the presence of mutant spores (table 12.1). Chimeras containing wild-type prestalk zones developed into fruiting bodies with 29% mutant spores while chimeras with wild-type prespore zones produced 8% mutant spores.

G Wild-type-Induced "Brainy" Mutant Development Across a Membrane Barrier

To determine if direct cell-to-cell contact was essential for wild-type inducement of mutant development or whether a diffusible component

Table 12.1    Grafting of Pre-stalk and Pre-spore Regions  
of Wild-type Slugs to NP551 Tight Aggregates

Plate	Slug Region	Mutant Aggregate	Fruiting Bodies Formed (Y/N)	% Mutant Spores
1	NC4-Tips (5)	NP551	Yes	29%
2	NC4-Tips (5)	—	Yes	0%
3	NC4-Backs (5)	NP551	Yes	8%
4	—	NP551 (5)	No	—
5	NP551 (5)	NP551	No	—

Numbers in brackets represent the number of chimeras tested.

mutant cells were present. If the wild-type cells preferred the prestalk zone, then the opposite would be true and in cases above 80% mutant, all spores produced would be mutant spores (Leach et al., 1973). The results suggest that both mutant and wild-type sort randomly to either zone; however, at high mutant levels, the majority of the wild-type cells may accumulate in the prestalk zone.

C Synergy of NC4 and NP551 after Segregated Development for 15 Hours

"Brainy" mutant and wild-type cells were allowed to develop separately in shaker culture for 15hr and then the two strains were allowed to develop in synergy as in the previous section. Mutant spores increased in a linear manner, relative to the number of 15hr mutant cells added and the results were nearly identical to that seen in figure 12.1 (data not shown). Therefore, it was concluded that synergy is only required after 15hr of development and mutant cells do not require interaction with vegetative or early stage aggregating wild-type cells to be induced to culminate. This further confirms the results in chapter 11 which showed that the "brainy" mutant developed aggregation competence.

D Effect of Glutaraldehyde-Fixed Wild-Type Cells and Wild-Type Ghosts on "Brainy" Mutant Development

Wild-type, strain NC4, cells were fixed for 30min in 0.25% glutaraldehyde in SORPHOS and then washed and resuspended in SORPHOS. Wild-type ghosts were prepared by the method of Sussman and Boschwitz (1975). Fixed wild-type cells or wild-type ghosts were mixed 1:1 with the "brainy" mutant and  $5 \times 10^7$  cells (or cells plus cell ghosts) were transferred to Millipore filters over SORPHOS soaked pads. Both 0hr and 15hr fixed wild-type cells were used in attempts to induce both 0hr and 15hr "brainy" mutant cells. All experiments produced negative results.

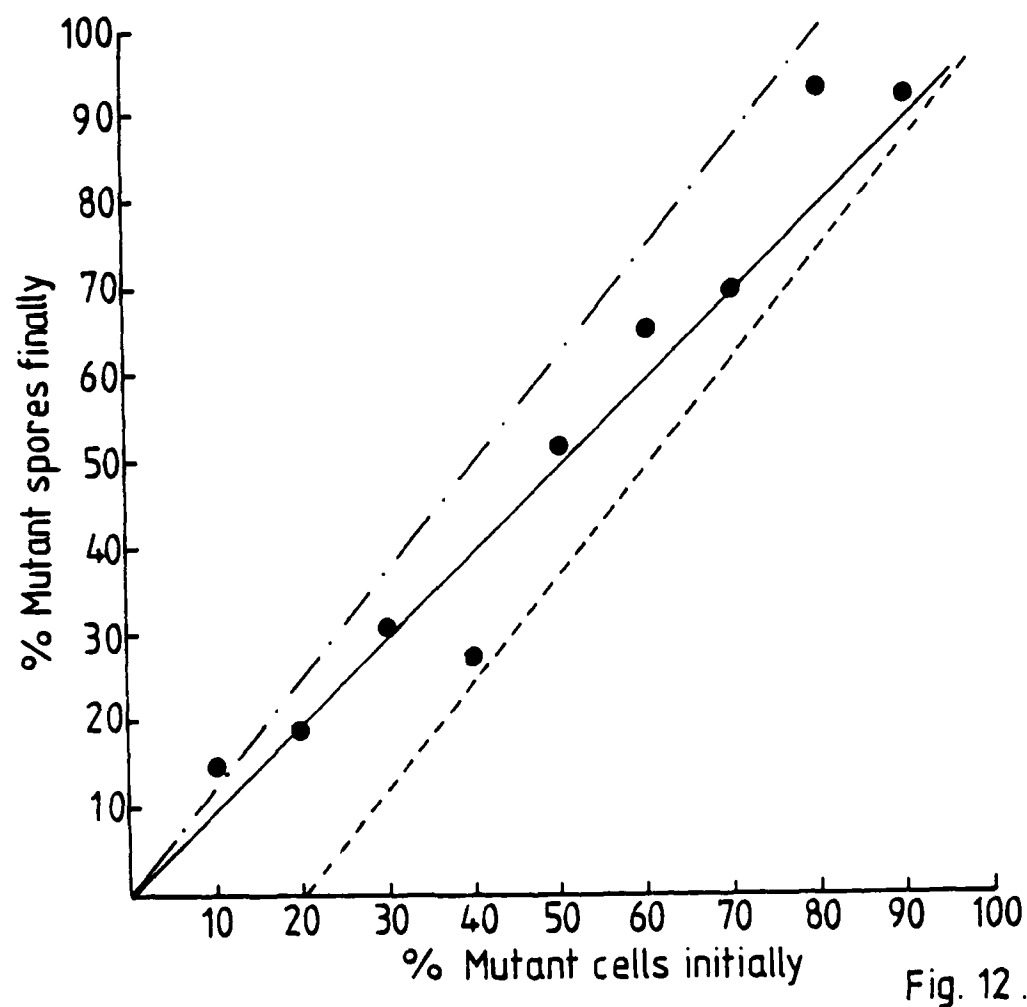


Figure 12.1 Synergistic Development of the  
"Brainy" Mutant

Vegetative amoebae of the wild-type strain, NC4, and the mutant strain, NP551, were mixed in a series of ratios at  $2 \times 10^8$  cells  $\text{ml}^{-1}$  and 0.1ml cell samples were deposited onto Millipore filters (25mm,  $0.45 \mu\text{m}$  pores) resting on non-nutrient SORPHOS buffered agar. Cell mixtures were allowed to develop for 48hr, then the frequency of spore genotypes was determined by culturing the spores on SM agar or SM agar with 2% methanol. The theoretical lines predict results if the wild-type showed pre-stalk preference (---), pre-spore preference (---), or no preference (—). This is a representative example from three separate experiments.

B Effect of Wild-type Cell Density on "Brainy" Mutant  
Synergistic Development

The KMMP agar was the optimum substratum for evaluating morphology of the formed chimeras, but for quantitative studies of mutant spore development, a Millipore substratum on non-nutrient SORPHOS buffered agar was employed. A spectrum of wild-type:mutant cell ratios were suspended at  $2 \times 10^8$  cells  $\text{ml}^{-1}$  and 0.1ml cell samples of each ratio were transferred to the surface of 25mm Millipore filters on the surface of 90mm agar plates and dried in a sterile flow hood until the cell surface had a matt appearance. Development was complete in 48hr and the fruiting bodies and aggregates on the surface of each filter were harvested into SORPHOS with 0.1% NP-40 as in section A above. The spore samples for each ratio were serially diluted and plated onto SM agar or SM agar containing 2% methanol with Klebsiella aerogenes as the food source. Only the methanol resistant NP551 spores germinated and formed clones on the methanol agar since NC4 is sensitive to growth on methanol; therefore, it was possible to determine the percent mutant and wild-type spores that form at each NC4:NP551 ratio.

The percentage of mutant spores increases in a linear manner with the increase in mutant vegetative amoebae initially deposited on the filter surface (figure 12.1). As shown, no mutant or wild-type spores develop when the cell mixture contains greater than 95% mutant cells. Apparently a minimum of 5% wild-type cells is required to induce mutant fruiting body formation.

The theoretical lines predicting the synergy results, if the wild-type shows prespore or prestalk preference, were derived in the following manner. Based on the fact that the tip region (prestalk region) of a tipped aggregate or slug constitutes 20% of the structure (front 1/5th, Raper, 1940), if the wild-type cells preferred the prespore region, then no mutant spores would form until greater than 20%

plated onto a variety of solid-substrata. To allow determination of the genotype of mature spores, a methanol resistant variant of the "brainy" mutant NP550 was selected from SM plates that contained 2% methanol. Thirty resistant clones were initially obtained and four of these methanol resistant variants were retained for further study. The variant used in most studies was NP551 which displayed the same "brainy" phenotype seen with NP550.

#### A Synergistic Development of the "Brainy" Mutant on KMMP Agar

Initial synergy experiments were conducted by allowing development of a mixed cell population on KMMP agar. The clear agar allowed easy assessment of the morphology of the chimeras that developed. Washed vegetative cells of the two strains were suspended at  $3 \times 10^7$  cells  $\text{ml}^{-1}$  in a series of ratios of wild-type (NC4):mutant (NP551) (10:1, 5:1, 3:2, 1:1, 2:3, 1:5, 1:10) and allowed to develop on KMMP agar. Ratios 2:3 and 1:5, provided the optimum cell interactions for mutant development. At these ratios, many double fruiting bodies were evident; these consisted of base fruiting bodies containing NC4 spores and second level fruiting bodies, emerging from the first sori, that contained NP551 spores, a phenomenon previously described by Bonner and Adams (1958). Spores from individual sori were harvested into SORPHOS containing 0.1% (v/v) NP-40 detergent to lyse any undeveloped cells that may exist in the sori (Devine et al., 1982). Clones from individual spores were obtained by spreader dilution and separate clones displayed the original phenotype of either the wild-type or the "brainy" mutant. This confirmed that synergy had occurred and mature spores of the developmentally defective "brainy" mutant had formed through synergistic aid from the wild-type.

were incubated for 3hr on pads soaked with  $\text{MgSO}_4$  and DTT and then transferred to normal SORPHOS-soaked pads (Rutherford and Brown, 1983).

All variations of this series of experiments failed to induce further development of the "brainy" mutant. In all cases, control experiments with NC4 cells under the various conditions produced normal wild-type fruiting bodies.

C Effect of Differentiation Inducing Factor (DIF) on  
"Brainy" Mutant Development

The putative morphogen DIF stimulates the differentiation of isolated amoebae into stalk cells (Town et al., 1976; Town and Stanford, 1979) and inhibits spore development thereby switching cells to stalk cell formation (Kay and Jermyn, 1983). Since the "brainy" mutant appeared unable to form tips, indicative of prestalk formation, the effect of DIF on NP550 cell development was tested.

D. discoideum strains NC4, V12M2 and NP550 were suspended in stalk salts solution (chapter 2) at three densities,  $2 \times 10^7$ ,  $5 \times 10^6$  and  $7.5 \times 10^3$  cells  $\text{ml}^{-1}$ . Two millilitre samples of each strain at each cell density were developed in 5cm tissue culture petri dishes in cell monolayers. Three hundred units of purified DIF (generous gift from Dr. R.R. Kay) were added to each petri plate and cultures were allowed to develop in a dark humidified chamber. After two days, cultures were examined by phase contrast microscopy for the presence of vacuolated stalk cells. Strain V12M2 was induced to produce greater than 90% stalk cells, whereas cells of strains NC4 and NP550 retained the appearance of vegetative amoebae.

12.2.2 Synergistic Development of the "Brainy" Mutant  
with Wild-Type Cells

Wild-type and "brainy" mutant cells were harvested from clearing mass plates, washed free of bacteria, mixed in various ratios and

an abnormal level of this attractant was produced in a mutant, perhaps tip formation would be inhibited. This possibility was tested by plating vegetative NP550 cells on SORPHOS buffered agar containing 0.15% activated charcoal in an attempt to establish a cAMP gradient within the developing aggregate. Under these conditions, the mutant did not proceed beyond its typical hemispherical aggregate stage of development.

B "Brainy" Mutant Development in Various Potentially-Inducible Chemical Environments

For this series of experiments, NP550 cells were allowed to develop on Millipore filters over buffer or chemical-soaked pads or on buffered agar with chemical additives. The following chemical environments were tested for their developmental effect:

- a) 5 $\mu$ M diethylstilboesterol (DES) in SORPHOS
- b) 5mM cAMP in SORPHOS
- c) Buffered agar, 20mM PO<sub>4</sub> pH 7.5, with 5mM NH<sub>4</sub>Cl
- d) SORPHOS buffered agar with 0.5mM propionic acid
- e) Agar buffered with 50mM Tris HCl pH 8.5, 0.1M MgSO<sub>4</sub>, 5mM DTT

The diethylstilboesterol (DES) experiment was conducted to determine if this steroid could mimic the effect of the stalk-cell differentiation inducing factor (DIF) (Gross et al., 1983). cAMP was tested because it may be an inducer of both stalk and spore differentiation (Kay, 1982). Furthermore, Gross et al. (1983) have shown that changing the cell environment by additions of NH<sub>4</sub><sup>+</sup> in the form of NH<sub>4</sub>Cl or H<sup>+</sup> in the form of propionic acid can induce cell-type specific development. The low Km form of phosphodiesterase may be the predominant enzyme form in pre-spore cells; therefore MgSO<sub>4</sub> and DTT were tested in an attempt to activate this enzyme form. Mutant cells

mutant may lack prestalk cells and therefore be unable to produce stalks. The results from the experiment which attempted to induce stalk cell differentiation with the differentiation inducing factor (DIF) were inconclusive since the wild-type NC4 amoebae also did not differentiate into stalk cells. Therefore, from this experiment, it was impossible to determine whether the "brainy" mutant has the genetic capability to produce stalk cells.

When viable wild-type developing cells interacted with mutant cells through direct cell-to-cell contact, mutant cells were induced to culminate and produce detergent resistant spores. Upon germination, these spores produced mutant clones that retained the "brainy" phenotype. Synergy experiments conducted across membrane barriers showed that synergy between mutant and wild-type cells occurred only when pores in the filter barrier were sufficiently large to allow cells in the lower lawn to transit the barrier and intermingle with the cells on the upper filter surface. The initial direct contact synergy studies showed that as few as 5% wild-type cells were sufficient to induce mutant spore development. Ray *et al.* (1979) estimate that it would be possible for 20 amoebae to contact a single central amoebae, therefore the 1:20, wild-type:mutant, ratio required for induced development may suggest that mutant cells must be in direct contact with wild-type cells to attain developmental competence. Results from the transmembrane studies are consistent with this interpretation.

A typical vegetative amoebae is approximately 10 $\mu$ m in diameter with a nucleus of approximately 5 $\mu$ m (Loomis, 1975). This may seem too large to transit these filters; however, the Millipore filter used was a cellulose ester depth filter (150 $\mu$ m thick) with a random matrix of flow channels. These filters are given pore size ratings even though pores of a specific size do not exist; therefore these Millipore filters would

possibly allow cell migration through this filter (Millipore catalogue). The Nucleopore filters used were only 10 $\mu$ m thick with distinct cylindrical pores. The results therefore suggest that amoebae can migrate through a 2.0 $\mu$ m pore. The negative result seen with the 0.6 $\mu$ m Nucleopore filter showed that a "diffusible factor" capable of inducing mutant development was apparently not produced by the NC4 lawn, since NC4 was unable to induce development even though the two cell types were separated by only one cell diameter (10 $\mu$ m). Additionally, the pores in this filter were sufficiently large to allow diffusion of large molecules.

Many workers have attempted to induce mutant development by wild-type cells across membrane barriers (Sussman, 1955; Sussman and Lee, 1955; Weber and Raper, 1971; Darmon et al., 1975; Town et al., 1976; West et al., 1983). Only two of these studies were able to demonstrate membrane diffusible components capable of affecting cell development. Darmon et al. found that cAMP from wild-type cells could diffuse across a cellophane barrier and induce development in aggregateless mutants. Town et al. reported a diffusible factor from high density cells on cAMP agar that could induce stalk differentiation in isolated cells. The compound was later identified as DIF (Town and Stanford, 1979). The fact that NC4 was unable to induce NP551 development in a similar manner across a thin porous filter suggests that the synergy effect seen here may instead involve: 1) a missing cell-type (e.g. prestalk cells), 2) a membrane bound compound, 3) a very labile molecule only functional through direct cell contact, or 4) an intracellular compound. The more promising possibilities are either a missing cell type or an intracellular compound. A membrane bound compound does not seem likely since neither glutaraldehyde fixed wild-type cells nor cell ghosts were able to induce further development of the "brainy" mutant. The fact that synergy did not occur across a thin porous membrane rules against a

labile compound, since the 0.6 $\mu$ m pores would possibly have allowed the thin filopodia of the two cell types to contact without mixing (Garrod and Born, 1971; Rifkin and Isik, 1984). The preliminary experiments that tested conditioned media and sonicated cell-extracts from wild-type cells may rule against the intracellular compound explanation. However, synergy experiments involving micelles from wild-type cells would further test this possibility. The results from the slug grafting studies do suggest that NC4 cells from the slug tip may be necessary for mutant development and therefore suggest that the "brainy" mutant may lack the organizing influence provided by prestalk cells (Raper, 1940; Rubin and Robertson, 1975; McWilliams and Bonner, 1979).

The results from the entire "brainy" mutant study (chapter 11, 12) suggest that this mutant may be a prespore-only mutant and therefore may be useful in cell sorting studies. However, the late appearance of the prespore specific markers implies that even the prespore cells may not fully develop in the absence of cell synergy with the wild-type. The general findings of this mutant study do indicate that synergistic interactions between cells may be a useful tool for elucidating mutant defects and cell-to-cell contact requirements during development.

## *Chapter 13*

### 13 GENERAL DISCUSSION

The working hypothesis established during the initial stages of this study was: folate serves as a chemoattractant message that mediates tipped aggregate formation in the cellular slime mould Dictyostelium discoideum. In the introduction, I proposed several criteria that had to be met in order to determine that tipped aggregate formation involved a folate-mediated communication event. These criteria included, 1) that folate receptors must be present at the tipped aggregate stage of development to receive the chemoattractant message, and, 2) that folate-induced cellular responses must also be demonstrated at this stage. The work described in this thesis verified that these two criteria are met and the results suggest that folate and folate receptors mediate cellular functions leading to tipped aggregate formation.

#### A Folate Receptors in Tipped Aggregate Formation

The folate receptors characterized in this study are in all likelihood the same receptors first detected by Wurster and Butz (1980), Van Driel (1981), and De Wit (1982). These folate receptors are extremely sensitive to minor modifications in the folate ligand structure and only those analogues with the complete folate backbone (pteridine ring, p-aminobenzoic acid, and glutamate) bind effectively. These receptors function as a single class that bind both folate and the deaminated analogue of folate, DAFA, with equal affinity. Contrary to the report of De Wit (1982) that described "non-specific" and "specific" folate receptors, no folate receptors were detected that were insensitive to DAFA competition (chapter 3). De Wit and Van Haastert (1985) described a major class of folate receptors that bind folate and DAFA with equal affinity, and they have termed this class, "A-sites". The folate receptors described in this thesis display binding

characteristics identical to this major receptor class. For simplification during the remainder of this discussion, I will also label the receptors I studied as "A-sites".

These A-sites appear developmentally regulated and decline from 50,000 sites cell<sup>-1</sup> during the vegetative stage to 10,000 by 12hr of development. This decline in receptor number corresponds with the loss of folate-stimulated chemotaxis during development (Pan et al., 1972). The residual pool of receptors that remain through 12hr of development may be the folate receptors that process the chemoattractant message during tip formation. It is feasible, though not confirmed in this study, that these cell surface receptors present at 12hr of development are the same as those folate receptors detected on isolated cytoskeletons (chapter 4). I propose that these cytoskeleton-linked receptors reflect the mechanism by which the folate receptors are actually "coupled" to the signal transduction pathway.

#### B Comparison of Folate Receptor Binding and the Chemotactic Response

Comparison of folate receptor specificity, assessed by the competition binding studies (chapter 5) and the chemotactic response assay (chapter 6), revealed several inconsistencies. The folate analogues, DAFA, methotrexate, and aminopterin bind to the folate receptor with an affinity equal to folate, yet DAFA does not stimulate chemotaxis in vegetative amoebae, and methotrexate and aminopterin induce a cell movement response more indicative of chemokinesis (Nandini-Kishore and Frazier, 1981; Varum and Soll, 1981) than chemotaxis. The pterin derivative, pterin-6-COOH, does not bind to the folate receptor and another derivative, pteric acid, binds with low affinity, yet both ligands stimulate chemotaxis in vegetative amoebae (chapter 5,6). The latter inconsistencies are explained if these pterin analogues are binding to the proposed pterin receptor (Pan et al., 1975;

Van Haastert et al., 1982c) and this pterin receptor is, in turn, mediating the observed chemotactic response. Interestingly, L-monapterin, an effective acrasin for D. lacteum (Van Haastert et al., 1982b), did not induce chemotaxis in vegetative cells, yet, L-monapterin induced a strong chemotactic response in cells developed for 12-24 hours. The lack of a chemotactic response by vegetative cells to L-monapterin is consistent with no binding to the folate receptor; yet, it is unclear why a significant L-monapterin response, mediated by the pterin receptor (as seen for pterin-6-COOH and pteric acid), was not evident.

The inconsistencies between receptor binding of folate analogues and chemotaxis toward these derivatives have been reported in other studies (Van Haastert et al., 1982c; De Wit, 1982; De Wit et al., 1985). This Dutch group has suggested that the A-sites are not the chemotactic receptor and have described another small pool of receptors (500-1500 sites cell<sup>-1</sup>) that may function in this role. This small pool of receptors, described by De Wit et al. (1985), are a class of high-affinity, slow-dissociating folate receptors, designated as B-sites. These B-sites are only detected after ligand binding and subsequent dissociation of the ligand from the low-affinity, fast-dissociating receptors (A-sites). The preliminary experiments which I performed to assay for B-sites through 12hr of development were inconclusive, so, I could not establish the presence or function of B-sites during D. discoideum development. However, as suggested in chapter 7, the B-sites may actually be A-sites that have, after ligand binding, been modulated via membrane protein or cytoskeletal interactions. The promising idea that folate receptors undergo modulation after ligand binding and that the true specificity of the receptor is only expressed in this modulated form, effectively explains the disagreement between specificity of folate receptor (A-site) binding and chemotaxis specificity. This

receptor modulation concept fits well with the finding that folate receptors are present on isolated cytoskeletons (chapter 4).

#### C Folate- and L-monapterin-induced Cellular Responses at the Tipped-Aggregate Stage of Development

The accumulation of actin in the Triton-insoluble cytoskeleton after folate stimulation of 12hr cells correlates with the appearance of tipped aggregates at this time of development (chapter 8). This confirms the preliminary report of McRobbie and Newell (1983) where cells were tested after shaker-culture development. The actin response in 12hr cells is a ligand-specific response since, of the many compounds tested, only folate and L-monapterin induce actin accumulation in these cells (chapter 8). The folate and pterin analogues, DAFA, lumazine, and xanthopterin, though similar stereochemically to folate or L-monapterin, failed to induce a similar response. It is interesting that L-monapterin possesses this stimulatory capability since this molecule does not bind to the folate receptor (chapter 5). However, the chemotactic response stimulated by L-monapterin in 12hr developing cells is consistent with the actin response data (chapter 6).

It is possible that the cellular response to L-monapterin has an evolutionary significance. Bonner (1982b) has hypothesized that there may be as many as eight different acrasins in the genus Dictyostelium. Three such acrasins have been identified: cAMP for D. discoideum, D. mucoroides, D. purpureum, and D. rosarium; L-monapterin for D. lacteum; and a folate analogue for D. minutum, (Konijn et al., 1967; Van Haastert et al., 1982b; Bonner, 1982b; De Wit and Konijn, 1983b). cAMP has also been implicated as an active chemoattractant in D. minutum tipped aggregate formation (Schaap et al., 1984; Schaap and Wang, 1985; Schaap, 1985). Bonner has proposed that the acrasin/acrasinase system in slime moulds is a complex system involving as many as twelve

interrelated substances. Therefore, for a species to evolve by converting from one acrasin system to another that was entirely unique would seem highly unlikely. In discussing evolution of acrasin systems, Bonner has observed that the potential acrasins, cAMP and folate (and perhaps pterins), are common to all cellular slime mould amoebae, and has stated that all that is needed to make them a unique acrasin is for their receptors to predominate on the cell surface at a key moment in development.

Throughout this study, I have assumed that since the folate-induced actin response correlates with tip formation, then folate must function in prestalk cell differentiation. Yet, of course, prespore cells also develop during this stage, and, therefore, a more consolidated view is feasible. Those species which utilize cAMP as the acrasin (D. mucoroides, D. purpureum, D. discoideum, D. rosarium) all demonstrate division of labour during development, possessing distinct prespore and prestalk zones (Bonner, 1982b). Since it is considered likely that distinct acrasins have evolved to allow isolation of species (Raper and Thom, 1941), I propose that the three chemoattractants folate, L-monapterin, and cAMP may function in a similar manner to isolate cell types during tip formation in these four species. Results from chapter 8 suggest that the folate- and L-monapterin-induced actin responses are mediated by distinct receptors. One could speculate that these two receptor types are segregated on the two alternative cell types. Such a possibility is consistent with Bonner's hypothesis. Several different chemoattractants could then function as acrasins (or perhaps morphogens) during development by a simple temporal or spatial control of receptor presence; such a mixed array of a limited number of acrasin systems is becoming increasingly apparent among Dictyostelium species.

#### D The 12-hour Actin Response and Cell Movement

It was accepted at the outset of this study that the folate-induced actin response was indicative of a cell movement event. Chemotaxis data, specifically data concerning the L-monapterin induced response, correlated temporally with the appearance of the L-monapterin stimulated actin response, thus relating the actin response with a cell movement event. However, there are distinct differences in the temporal pattern of the folate-induced actin response in vegetative cells compared to the response in 12hr cells. In early stage cells, the actin response (chapter 8) clearly includes a reduction of actin in isolated cytoskeletons after the initial 3-5sec peak of accumulation, termed the "trough", which is temporally correlated with the cell "cringe" (McRobbie, 1984). This dramatic reduction in actin is not evident in the folate- or L-monapterin-induced responses in 12hr cells. The "cringe" may be a phenomenon seen only in amoeboid, vegetative cells and not in the more rounded and perhaps more rigid developing cells. If, as suggested by (McRobbie and Newell, 1983), the rapid initial actin-peak (3-5sec) is a reflection of pseudopod formation then one might expect that this response would be lost in developing cells if pseudopod formation is reduced in cell aggregates. However, the folate- and L-monapterin-induced actin peak at 5-10sec is possibly more pronounced in developing cells. An alternative explanation should perhaps be considered.

Ligand-receptor interactions may induce receptor modulation and result in attachment of the receptor to the cytoskeleton, perhaps with the involvement of an effector molecule (chapter 4, chapter 7, Jacobs and Cuatrecasas, 1976; Schechter and Bothwell, 1981; Jesaitis et al., 1984). These studies and others suggest that receptors may become

attached to the cytoskeleton after ligand-stimulation, resulting in an increase in receptor-ligand affinity. In mammalian systems, perhaps through a similar mechanism, divalent antibodies and cross-linking lectins (Concanavalin A and wheat germ agglutinin) can also induce an increase in receptor-ligand affinity and association of the receptor with the cytoskeleton (Shechter et al., 1979; Vale and Shooter, 1982). Condeelis (1979) has shown in Dictyostelium amoebae that after specific binding of ConA to the cell surface, and upon Triton X-100 cell lysis, a large amount of the cell's actin and myosin copurifies with the ConA bound plasma membrane receptors. This worker has demonstrated that ConA induces membrane protein patching and capping on the cell surface and has suggested that membrane-associated actin and myosin filaments are responsible for the accumulation of ConA-receptor complexes into patches and caps on the cell surface.

The ligand-induced actin response may, therefore, not be entirely a cell motility related phenomenon. It seems that this response may indicate not only actin involvement in cell movement but also actin involvement in receptor modulation, defined by Edelman (1976) as "any change in the structure, pattern, or dynamic state of receptors at the cell surface." Consequently, the actin response seen in 12hr cells could be indicative of only receptor-cytoskeleton interactions and not cell movement. I envisage that folate-induced receptor interactions with the cytoskeleton would stabilize the cytoskeleton which, in turn, could result in an increase in actin associated with the cytoskeleton upon Triton X-100 cell lysis. In light of this interpretation, the folate- or L-monomapterin-induced 12hr actin response could feasibly be a cytoskeleton-mediated receptor-modulation event involved in transduction of a specific message which directs a cell-type specific event leading to developmental regulation and pattern formation.

E The Role of Calcium in Signal Transduction in 12hr Developing Cells

The drug, TMB-8, known to block calcium mobilization, effectively inhibits the actin response and the sensitivity of the actin response to this drug is similar to that seen for aggregation and cAMP-induced cGMP accumulation (Europe-Finner and Newell, 1984). However, as seen in TMB-8 studies with thymocytes, TMB-8 is a potent inhibitor of cellular respiration in Dictyostelium discoideum. When cells, inhibited by TMB-8, are treated with succinate or calcium, a partial restoration of respiration is observed, and if succinate and calcium are added together, respiration rates return to normal levels. Calcium and succinate seem to stimulate renewed respiration in a synergistic manner which suggests that TMB-8 may disrupt calcium-related mitochondrial function as well as block NADH dehydrogenase (Brand and Felber, 1984). The finding that TMB-8 inhibits respiration when applied at a dose that is one-tenth that required to inhibit ligand-induced cellular responses, suggests that the TMB-8 effect on calcium-requiring signal transduction events is distinct from the effect on respiration. Yet, the similar protective effect of added calcium on both the actin response and cellular respiration prevents any segregation or interpretation of these calcium effects.

F The cGMP Response and the Actin Response May Be Mediated by Separate Signal Transduction Pathways

Elevated intracellular cGMP levels, indicative of a cell movement-related signal transduction event, are not evident after folate stimulation of 12hr cells of either the wild-type strain, NC4, or the "streamer" mutant. This result is consistent with the signal transduction model of McRobbie and Newell (1984b) which proposes that the actin response pathway is independent from the pathway that activates

guanylate cyclase. Alternatively, if the 5sec actin response is related to receptor/cytoskeleton interactions and receptor modulation, then this actin mediated event could conceivably precede cGMP on a common signal transduction pathway. If this was so, then the lack of a cGMP response in folate-stimulated 12hr cells would suggest that folate was not inducing cell movement in these cells.

An intriguing fact is that the McRobbie and Newell model is compatible with the well-defined transduction mechanism that involves the hydrolysis of phosphoinositides to diacylglycerol (DG) and inositol triphosphate ( $IP_3$ ) (reviewed by Berridge, 1984). This bifurcated pathway, with cGMP production induced by DG catabolism and calcium mobilization induced by  $IP_3$ , could effectively mediate the apparently divergent pathways leading to cGMP production (via DG) and the actin response (via  $IP_3$ ) in Dictyostelium.

#### G The "Brainy" Mutant May Contain Only Prespore Cells

Results from the characterization of the "brainy" mutant suggest that developing aggregates of strain NP551 may contain only prespore cells (chapter 11). The mutant displays the normal cell responses and developmentally regulated behaviour seen in wild-type cells as they attain aggregation competence, despite the abnormal "brainy" phenotype manifested at the onset of aggregation. One feature that implies that the mutant may produce only prespore cells is the failure of the mutant to manifest tipped aggregates during development on a solid substratum. Additionally, NP550 produces prespore vesicles and prespore- and spore-specific proteins; the accumulation of prestalk and stalk proteins, though not easily detected by silver staining (Morrissey, et al., 1984), was not evident during development. These spore specific markers, though delayed in their appearance during development, are indicative that this mutant possesses the genetic capability to produce normal prespore and spore cells. This genetic capability is further

substantiated by the discovery that NP551 cells can be induced to produce detergent-resistant spores when developed in synergy with as few as 5% wild-type cells. Preliminary experiments which involved grafting of wild-type slug tips to mutant aggregates, suggest that the wild-type tip cells induce mutant spore development more effectively than wild-type prespore cells. These experiments and the transmembrane developmental synergy tests, which verified that mutant cells are only induced to develop mature spores when in direct contact with wild-type cells, imply that the inducing component supplied by the wild-type cells may be a specific cell-type, perhaps prestalk cells.

#### H Further Outlook and Future Research

The likely involvement of the cytoskeleton in receptor modulation and the premise that the actin response actually reflects ligand-induced receptor/cytoskeleton interactions certainly warrants further investigation. Identification of the folate receptor and production of antibodies against the purified receptor would perhaps be an initial step in such an investigation. A method, potentially useful for labelling the folate receptor, has been developed to activate the methotrexate or folate molecule and then covalently link the molecule to proteins via a peptide bond (Kulkarni et al., 1981; Ananthanarayanan, 1984; Bachas et al., 1984).

The discovery that L-monapterin induces an actin response at 12hr of development may prove interesting. Future studies of the developmental regulation of the pterin receptor and of the pterin degradation enzyme could further develop the hypothesis that a limited number of acrasin systems are employed in temporally and spatially diverse ways during Dictyostelium development.

Additional experiments that utilize internalized fluorescent dyes to study cell-sorting in migrating slug chimeras, constructed from wild-

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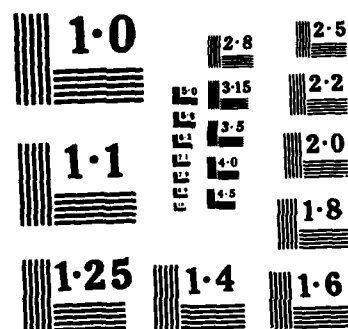
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type and "brainy" mutant cells, would be one way to study this mutant further. Other cell sorting experiments that mix the "brainy" mutant cells and cells from the temperature-sensitive mutants that develop predominantly prestalk cells, described by West et al. (1982,1983) could also provide fruitful results. Preliminary experiments have shown that the 12hr developing "brainy" mutant cells are very responsive to chemoattractant stimulation by L-monapterin. Use of the "brainy" mutant in actin response studies may further advance the prospect of an L-monapterin role in cell-type specific cell differentiation.

It is becoming increasingly evident that the diacylglycerol and inositol triphosphate (DG/IP<sub>3</sub>) pathways comprise a common mechanism that is central to calcium-related signal transduction in most eukaryotic cells. I predict, therefore, that it will be worthwhile to investigate the roles of DG and IP<sub>3</sub> as potential second messengers in the transduction-related cGMP and actin accumulation responses in Dictyostelium discoideum.

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## *Appendices*

# APPENDIX I

## CHEMICALS

## SOURCE

Purified Agar	
Technical Agar No. 3	
Yeast Extract	Oxoid Ltd, Basingstoke, Hampshire
Adenosine 3':5'-cyclic monophosphate	
p-Aminobenzoic Acid	
Brilliant Blue R (Coomassie Blue)	
Bromphenol Blue	
Carbonic Anhydrase	
Deoxyribonuclease I (Bovine Pancreas)	
Dithiothreitol (DTT)	
EGTA (ethyleneglycol-bis-( $\beta$ -aminoethyl ether) N,N,N',N',-tetraacetic acid)	
EDTA (ethylene-diamine-tetraacetic acid)	
Folic Acid	
B-galactosidase	
Glycine (ammonia free)	
Glutamic Acid	
Imidazole (Grade I)	
Mineral Oil, light	
Mineral Oil, heavy	
Phosphorylase B	
Rabbit Muscle Actin	
Rabbit Muscle Myosin	
Ribonuclease A (Type 1A)	
Trizma Base	Sigma Chem Co., Poole, Dorset
Calcium Oxide (Specifically purified)	
37% Formaldehyde	
50% Glutaraldehyde	
Nonidet P-40 (NP-40)	
PPO (2,5-diphenyloxazole)	
Silver Nitrate	
Sodium Dodecyl Sulphate (Specifically Purified)	BDH Chem Ltd, Poole, Dorset
Acrylamide (>99.9% pure)	
Ammonium Persulphate	
Bio-Rad Protein Assay Dye Reagent	
Methylene-bis-acrylamide	
TEMED (N,N,N',N'-tetramethylene diamine)	
Triton X-100 (Electrophoresis purity)	Bio-Rad Lab Ltd, Watford, Hertfordshire
Bovine Serum Albumin	
TMB-8 ((8-diethylamino)octyl- 3,4,5-trimethoxybenzoate HCl)	Cambridge BioScience, Hardwick Cambridge

Pteric Acid	
Lumazine	
L-monapterin	
8-Aza-guanine	
Amethopterin (Methotrexate)	Fluorochem Ltd, Glossop, Derbyshire (Agent for Fluka AG)
Urea (Ultra pure)	Schwartz-Mann (Becton & Dickerson, Oxford)
Ampholytes (pH 5-7, pH 3.5-10)	LKB Inst. Ltd, South Croyden, Surrey
Triton X-100 (Scintillation Grade)	
Unisolve I Scintillant	Koch-Light Laboratories Ltd Colnbrook, Slough, Berkshire
[8- <sup>3</sup> H] Adenosine 3':5' cyclic monophosphate (cAMP)	
[7,9,3',5'- <sup>3</sup> H]Folic Acid	
cGMP Radioimmunoassay Kit	Amersham International PLC, Amersham, Buckinghamshire
Silicone Oils (AR20, AR200)	Wackerchemie, Munich, FRG
Dow Corning Silicone Fluid 550	Hopkin and Williams, Chadwell Heath, Essex
Xanthopterin	
Pterin-6-carboxylic Acid	
Aminopterin	
p-Aminobenzoyl-Glutamic Acid	Aldrich Chemical Co.
2,4 Hydroxy-deamino Folic Acid (DAFA)	Gift, Mr. Vitrus Lau American Cyanamid Co. Wayne, N.J. 07470
Dichloro-methotrexate	
2,4-Diamino-5-(3',4'-dichlorophenyl) -6-methyl pyrimidine	
Triazinate	Gift, Dr. Robert Engle Dev Therapeutic Program National Cancer Institute
DIF (Differentiation Inducing Factor)	Gift, Dr. R.R. Kay Laboratory of Molecular Biology Cambridge

## APPENDIX II

EQUIPMENT	SOURCE
Gilson tips, 200 $\mu$ l, yellow Petri Dishes, 9cm, Triple Vent Plastic Disposable Tubes (6mm x 12cm)	Sterilin Ltd, Teddington Lock Middlesey
Borosilicate Glass Tubing (1.0-1.2 internal dia, 0.2mm wall)	Custom made, Plowden and Thompson Stourbridge, West Midlands
Silastic Tubing (Medical Grade) (0.76 x 1.65mm)	Dow Corning (McCarthy Surgical Supplies)
Cristaseal Sealing Compound Platon Flow Meter	Gallenkamp
Silica Gel (1-3mm, 6-16 mesh)	Fisons Scientific Loughborough, Leistershire
Millipore Filters, Prefilters	Millipore
Eppendorf tubes, 1.5ml Eppendorf tubes, 400 $\mu$ l longform Gilson Tips, 1000 $\mu$ l, Blue	Anderman
LKB Wallac 81000 Scintillation Counter LKB Rack Beta (Apple IIe assisted)	LKB Inst. Ltd, South Croyden Surrey

# APPENDIX III

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10 LPRINT SPC(20)"DISTRIBUTION-FREE PLOTS"
15 LPRINT SPC(10)"CALCULATION OF Kds AND NUMBERS OF BINDING SITES"
20 PRINT "ENTER DATA SET NAME OF INPUT DATA"
30 INPUT DSN$
40 DIM S(100),V(100),M(1000),K(1000),P(100),U(1000),W(1000),A(100),B(100),C(100),D(100)
42 OPEN "I",#1,DSN$
43 INPUT #1,N
44 INPUT #1,J
45 LPRINT N
46 LPRINT J
50 FOR I=1 TO N
60 INPUT #1,A(I),B(I),C(I),D(I)
70 LPRINT A(I),B(I),C(I),D(I)
100 S(I)=(D(I)-(A(I)*D(I)/C(I))-(B(I)*D(I)/C(I)))
110 V(I)=(A(I)*D(I)/C(I))-(B(I)*D(I)/C(I))
120 P(I)=S(I):S(I)=1/V(I):V(I)=P(I)/V(I)
130 NEXT I
140 L=1
145 W=2
150 FOR I=1 TO N-1
160 FOR H=W TO N
170 K(L)=-(V(I)-V(H))/((V(H)/S(H))-(V(I)/S(I)))
180 M(L)=-(V(I)/S(I))*K(L)+V(I)
185 PRINT "K(L)"K(L),"M(L)"M(L),"I" I,"H" H
190 L=L+1
200 NEXT H
205 W=W+1
210 NEXT I
215 I=L-1
217 W=2
220 FOR L=1 TO I-1
230 FOR H=W TO I
240 IF K(L)>K(H) GOTO 271
250 T=K(L)
260 K(L)=K(H)
270 K(H)=T
271 IF M(L)>M(H) GOTO 280
272 T=M(L)
273 M(L)=M(H)
274 M(H)=T
280 NEXT H
285 W=W+1
290 NEXT L
295 PRINT "K(L)","V(L)" "SORTED INTERCEPTS"
300 FOR L=1 TO I
310 PRINT K(L),M(L)
320 NEXT L
325 L=INT(L+.5)
330 X=L
340 X=(X/2)
345 L=INT(L/2)
350 IF(X-L)<=.1 GOTO 395
360 Z=X+(X-L)
365 L=Z
366 U1=1/K(L)
367 W1=M(L)*U1
369 U3=U1*XJ
375 LPRINT,"AVERAGE NUMBER OF BINDING SITES="U3
380 LPRINT,"AVERAGE Kd="W1
390 GOTO 430
395 Y=(K(L)+K(L+1))/2
400 O=(M(L)+M(L+1))/2
410 U2=1/Y

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411 W2=CXU2
415 LPRINT,"AVERAGE NUMBER OF BINDING SITES="U2XJ
425 LPRINT,"AVERAGE Kd="W2
428 GOTO 475
430 FOR L=1 TO I
438 U(L)=K(L)-K(Z)
452 W(L)=M(L)-M(Z)
454 W(L)=W(L)XU1
456 LPRINT,"NUMBER OF BINDING SITES=";(U1+U(L))XJ;"Kd=";W1+W(L)
460 NEXT L
470 END
475 FOR L=1 TO I
480 PRINT K(L)-Y,M(L)-W
485 U(L)=(K(L)-Y)
486 W(L)=M(L)-O
488 W(L)=W(L)XU2
492 LPRINT,"NUMBER OF BINDING SITES=";(U2+U(L))XJ;" Kd=";W2+W(L)
494 LPRINT
495 NEXT L
500 END

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## *Publications*

#### PUBLICATIONS

- Tillinghast, H.S., Jr and Newell, P.C. (1984)  
Retention of folate receptors on the cytoskeleton of Dictyostelium discoideum during development, FEBS Lett. 176, 325-330.
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